AD____________________

Award Number: W81XWH-06-1-0537

TITLE: Identification of Tumor Rejection Antigens for Breast Cancer Using a Mouse Tumor Rejection Model

PRINCIPAL INVESTIGATOR: Mary L. Disis, M.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98105

REPORT DATE: May 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-05-2009	**2. REPORT TYPE** Final	**3. DATES COVERED** *(From - To)* 28 Apr 2007 - 27 Apr 2009

4. TITLE AND SUBTITLE
Identification of Tumor Rejection Antigens for Breast Cancer Using a Mouse Tumor Rejection Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0537

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mary L. Disis, M.D.

E-Mail: ndisis@u.washington.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Washington
Seattle, WA 98105

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Abstract provided on next page.

15. SUBJECT TERMS
Tumor rejection, antigens, mouse model

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	31	**19b. TELEPHONE NUMBER** *(include area code)*

ABSTRACT

Pre-menopausal patients whose breast cancers are, in general, estrogen receptor (ER) negative and biologically more aggressive, are particularly in need of novel therapeutic interventions. A breast cancer vaccine to prevent relapse after conventional treatment would be of enormous clinical value. Several cancer vaccine studies have demonstrated that breast cancer patients can develop tumor antigen specific immune responses after a vaccine is given. Most of these approaches target a single immunogenic protein or antigen thus limiting the vaccine to those patients whose tumor expresses that antigen. We propose the development of a multi-antigen vaccine that could potentially benefit any ER negative breast cancer patient; the category of patient at highest risk of relapse. Several high throughput antigen discovery tools have been developed that have greatly helped the identification of immunogenic proteins in breast cancer patients. One such technique, SEREX (serological analysis of cDNA expression libraries) identifies tumor antigens based on spontaneous antibody immunity that can occur in breast cancer patients. To date, over 2,000 tumor antigens have been identified from a variety of cancers using SEREX. In fact, so many tumor antigens have been identified a major question facing tumor immunologists today is- which antigen will induce anti-tumor immunity? Investigators evaluating immunity to leukemia have recently utilized patients who develop an anti-tumor response after immunotherapy to identify true "tumor rejection" antigens, that is, those immunogenic proteins which, if targeted, would induce an anti-tumor response. This work was possible because one of the treatments of relapsed leukemia is immunotherapy using donor lymphocyte infusions. The investigators could identify which immunogenic proteins were associated with the development of a remission after immunotherapy. Unfortunately, immunotherapy has not yet advanced to a stage in the treatment of breast cancer where we can reproducibly induce tumor regression. We have recently determined that the neu transgenic (neu-tg) mouse can serve as an excellent model for identifying human breast cancer antigens. These mice were genetically engineered to develop breast cancer that is almost identical to human breast cancer. The breast cancers that occur in these mice are ER negative and drug resistant mimicking pre-menopausal breast cancer in women. Preliminary experiments have shown that neu-tg FVB/N mice may share the same pool of tumor antigens with breast cancer patients. Our goal in this proposal is to identify antigens that are associated with tumor rejection. Whereas this study would not be possible in humans, we have recently established a tumor rejection model by implanting the mouse tumors derived from neu-tg mice into the parental FVB/N mouse, who are identical in every way except were NOT engineered to develop breast cancer. In our model, none of the parental FVB/N mice develop tumor while all of the neu-tg mice that received tumor implantation succumb to their disease despite having endogenous immunity to some proteins expressed by the tumor. Interestingly, the tumor rejection that occurs is not solely mediated by immunity to neu- the major cause of the cancer (similar to human HER-2/neu). We proposed to use subtractive SEREX, a method established in the laboratory to screen for antigens that are specifically induced by tumor rejection. The tumor antigens that have immunogenic human homologues will be further studied by using them to vaccinate the neu-tg mice to see if such a vaccine will prevent the cancers. The human homologues of these proteins, identified as described in this proposal, will be the basis for a multi-antigen vaccine to prevent breast cancer relapse in pre-menopausal patients with ER negative breast cancer.

Table of Contents

Introduction

The purpose of this study is to identify tumor rejection antigens using mouse tumor rejection models. Although a number of tumor antigens have been identified in cancer patients, it remains a challenge to identify therapeutically relevant tumor rejection antigens. Previous studies in our lab have demonstrated that the tumor antigen repertoire in tumor-bearing neu transgenic mice has great similarity to the antigen repertoire in breast cancer patients. Immune response-mediated tumor regression, although difficult to achieve in cancer patients, have been reported in mice. The goal of the proposed study is to identify tumor rejection antigens using mouse tumor rejection models. The study has three specific aims: (1) to determine the antigen repertoire induced by tumor rejection in FVB/N mice; (2) to identify the human homologues of the candidate rejection antigens and determine their immunogenicity; and, (3) to examine the *in vivo* tumor protection effect of vaccination with plasmids encoding tumor rejection antigens in neu-tg FVB/N mice. During the three funding years (2006-2009), we have successfully identified the antigen repertoire induced by tumor rejection in FVB/N mice. The human homologues of the candidate rejection antigens have also been evaluated. The potential tumor protection effect of the identified novel antigens have been tested in both implanted and spontaneous tumor models.

Key Research Accomplishments

A panel of 10 tumor rejection antigens have been identified in mice rejecting tumors. Six of them have immunogenic human homologues. Vaccination using these antigens has tumor protection effect, in both implant and spontaneous tumor setting. A multi-antigen plasmid DNA vaccine is more potent than single antigen vaccines in protecting mice from developing tumor.

Specific Aim 1: to determine the antigen repertoire induced by tumor rejection in FVB/N mice.

During the first funding year (2006-2007), we established two mice tumor rejection models. One of them is by injecting the tumor cells from neu transgenic mice into parental mice and resulting in neu-mediated rejection (Fig. 1). The second is by depleting T regulatory cells using IL2-immunotoxin [1]. SEREX screening by comparing the serum antibody repertoire prior to and post to tumor rejection identified a panel of 10 tumor rejection antigens, including FxyD3, Cep290, Ctnna1, Tln1, Hsp40, GPIap1, Tnfaip3, Hnrpl1, Tmem57, and Mtv1. This antigen panel is totally different from the antigen panel we previously identified in tumor-bearing mice [2], indicating that the antigens mediating the tumor destructive immunity may be distinct from the antigens that induce immune tolerance to tumor (Table 1). The expression profile of the rejection antigens were examined using real time RT-PCR. As shown in Fig. 4, some antigens are expressed in normal tissue as well as tumors (Fig 4A-D). FxyD3 and Mtv1 have a restricted expression pattern with little expression in normal tissues (Fig. 4E-F).

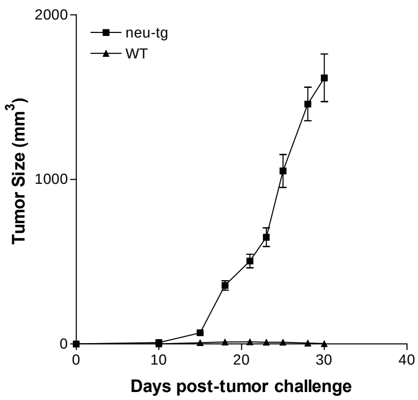

Figure 1. FVB/N tumor rejection model. One million MMC tumor cells were injected subcutaneously into wild type (WT) or neu transgenic (neu-tg) FVB/N mice. Shown are tumor size in neu-tg mice (■), and parental mice (▲).

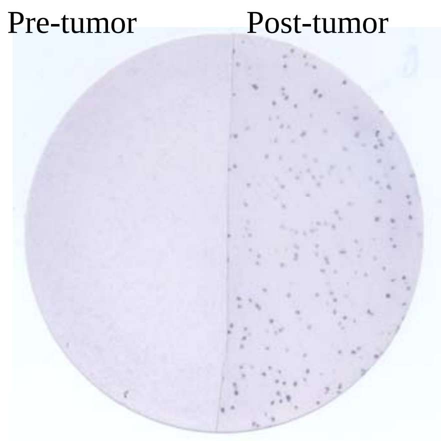

Figure 2. SEREX can be used to identify tumor rejection an tigens. Shown is a representative SEREX blot showing that Mtv1, one of the identified antigens, is only reactive to post-rejection serum.

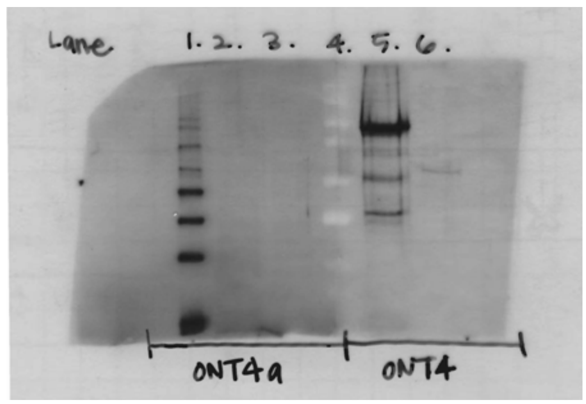

Figure 3. Western blo t verifying the an tibody response id entified in SEREX. ONT4a is pre-tumor serum from a neu-tg mouse, and ONT4 is the post-tumor rejection serum from the same mouse.

Table 1. Comparison of tumor antigens identified in tumor-bearing mice and tumor-rejecting mice

Antigen	No. of clones	Gene Symbol	Immunogenic Human Homologue	Human SEREX clone
Mouse mammary tumor virus	6	Mtv1		
Catenin (cadherin-associated protein) alpha 1	4	Ctnna1	CTNNA1	NY-REN-13
Heat shock protein 40	2	Hsp40	HSP40	MO-BC-1001
Transmembrane protein 57	2	Tmem57		
Centrosomal protein 290 kDa	1	Cep290	CEP290	NY-TLU-66 HOM-Ov1-214
FXYD domain containing ion transport regulator 3	1	Fxyd3		
Talin 1	1	Tln1	TLN1	NY-BR-88
Heterogenous nuclear ribonuclear protein L-like	1	Hnrpl1	HNRPL1	MO-OVA-131
GPI-anchored membrane protein 1	1	GPIap1		
TNF alpha-induced protein 3	1	Tnfaip3	TNFAIP3	MO-OVA-200

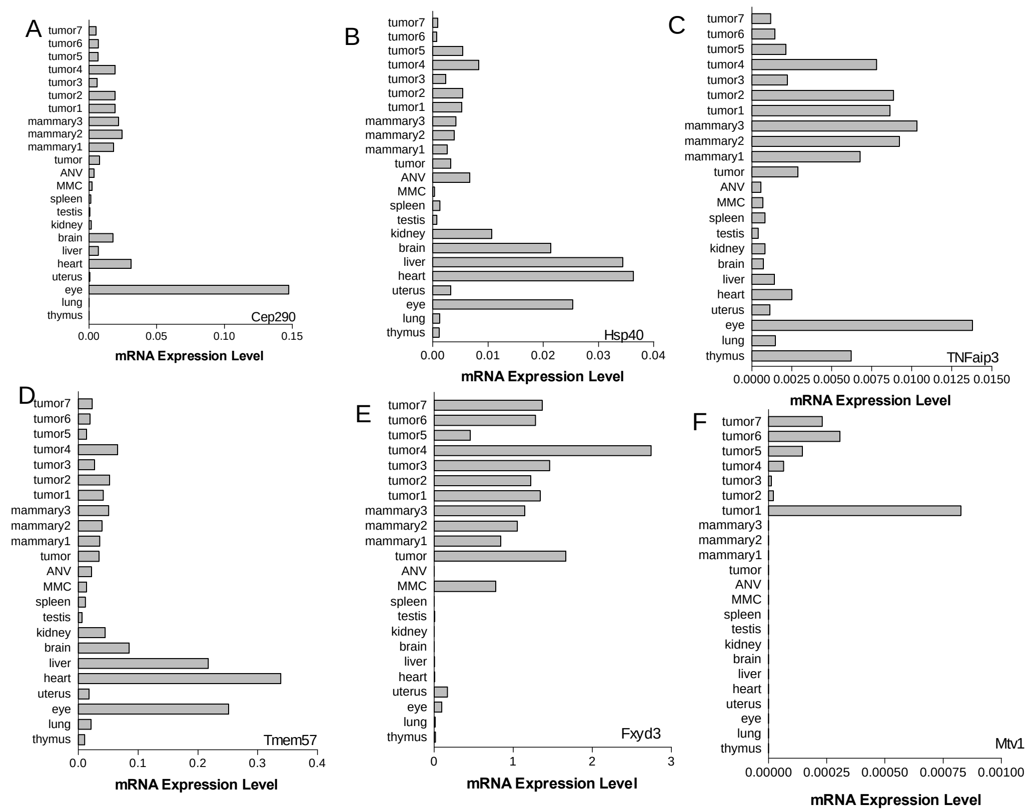

Figure 4. SEREX-identified tumor rejection antigens (TRA) have different expression patterns. **A-D**: Four of the TRAs are expressed in normal tissue in addition to tumor tissues. **E**: Fxyd3 has a restricted expression in mammary tissue and tumor. **F**: Mtv1 is exclusively expressed in tumor tissue.

Specific Aim 2: to identify the human homologues of the candidate rejection antigens and determine their immunogenicity.

The human homologues of the candidate antigens have been identified (Table 1). By searching published literature and database mining, we have found that six out of the ten tumor rejection antigens have immunogenic human homologues, including Ctnna1, Hsp40, Cep290, Tln1, Hnrpl1, and Tnaaip3 (Table 1). The potential therapeutic and prognostic value of these antigens will be evaluated in future studies.

Specific Aim 3: to examine the *in vivo* tumor protection effect of vaccination with plasmids encoding tumor rejection antigens in neu-tg FVB/N mice.

Multiple vaccination experiments were carried out to test the potential tumor protection effect of the identified antigens. The vaccines were tested in both implanted tumor and spontaneous tumor setting.

<u>3.A. Vaccination with tumor rejection antigens inhibits the growth of **implanted tumor**.</u>

3.A.1. Vaccination using tumor rejection antigens but not other tumor antigens has tumor protection effect. Female neu transgenic mice received plasmid DNA encoding the different tumor antigens. Plasmid DNA encoding the empty vector (pBK-CMV) was included as negative control. Plasmid DNA encoding the intracellular domain of human HER2 (hICD), was included as positive control. Mice were given three vaccines intradermally (50ug plasmid DNA per vaccine), two weeks apart. Tumor challenge using a syngeneic tumor cell line was given at two weeks after the third vaccine. The tumor growth was measured twice a week using venier calipers. As shown in Fig. 5, vaccination using Mtv1, FxyD3, and

Cep290 had tumor protection effect. In contrast, vaccination targeting the antigens we previously identified from tumor-bearing mice, such as Swap70 and Rock1, did not have tumor protection effect (Fig. 6). To further confirm the observation that antigens from tumor-rejecting mice but not tumor-bearing mice had tumor protection effect, we tested the 2 groups of antigens side-by-side in one experiment. As shown in Figure 7, the tumor growth curves separated into two groups. The first group of mice had the same tumor growth rate as the mice received empty vector (negative control). These included mice received Rock1, Gsn, Swap70, and Eprs, all of which were identified from tumor-bearing mice. The second group of mice had the same growth rate as the mice received hICD (positive control). These include mice received FxyD3, Tmem57, Mtv1, and Cep290, all of which were identified from tumor rejection mice. Altogether, these experiments demonstrate that vaccination targeting antigens identified from tumor rejection mice but not antigens identified from tumor-bearing mice had tumor protection effect.

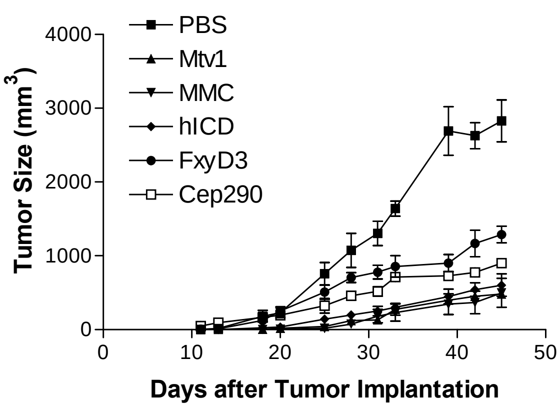

Figure 5. Vaccination targeting tumor rejection antigens, Mtv1, FxyD3, or Cep290, resulted in tumor protection. Mice (3 per goup) were vaccinated with plasmid DNA encoding Mtv1, FxyD3, Cep290, or hICD, or irradiated whole tumor cells at day -42, -28, and -14. Live MMC cells were given subcutaneously on day 0.

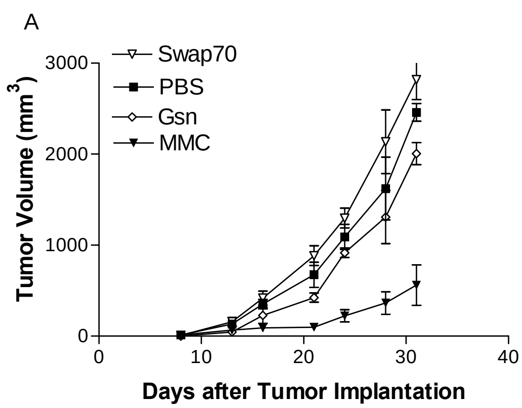

Figure 6. Vaccination targeting tumor antigens identified from tumor-bearing mice, Swap70 and Gsn, did not have tumor protection effect. Mice (3 per goup) were vaccinated with plasmid DNA encoding Swap70 or Gsn, or irradiated whole tumor cells at day -42, -28, and -14. Live MMC cells were given subcutaneously on day 0.

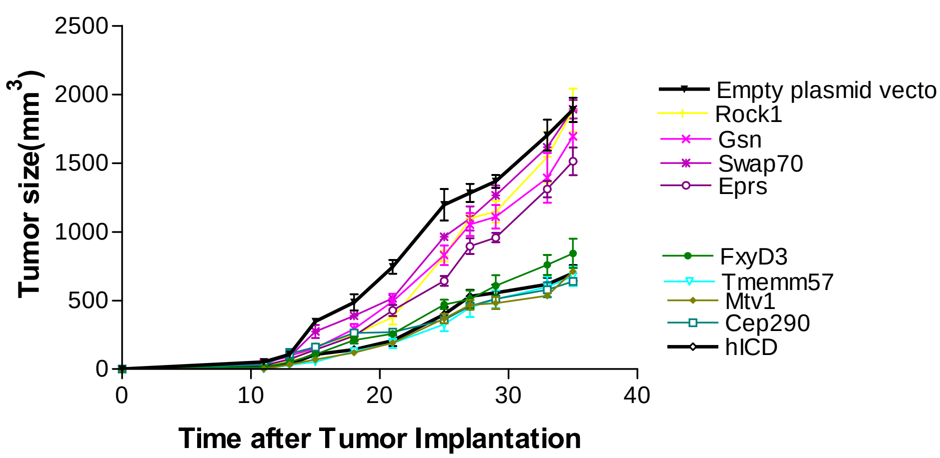

Figure 7. Vaccination targeting antigens identified from tumor-rejecting mice but not tumor-bearing mice had tumor protection effect. Four tumor rejection antigens (FxyD3, Tmemm57, Mtv1, Cep290) and 4 non-tumor rejection antigens (Rock1, Gsn, Swap70, Eprs) were examined side-by-side in one experiment. Empty plasmid vector was included as negative control and hICD was included as positive control.

combination of antigens worked better than single antigen. The tumor protection effect of 2 antigens (Mtv1+FxyD3) was better than either agent alone. The combination of three antigens (Mtv1+FxyD3+hICD) provided the most tumor protection effect.

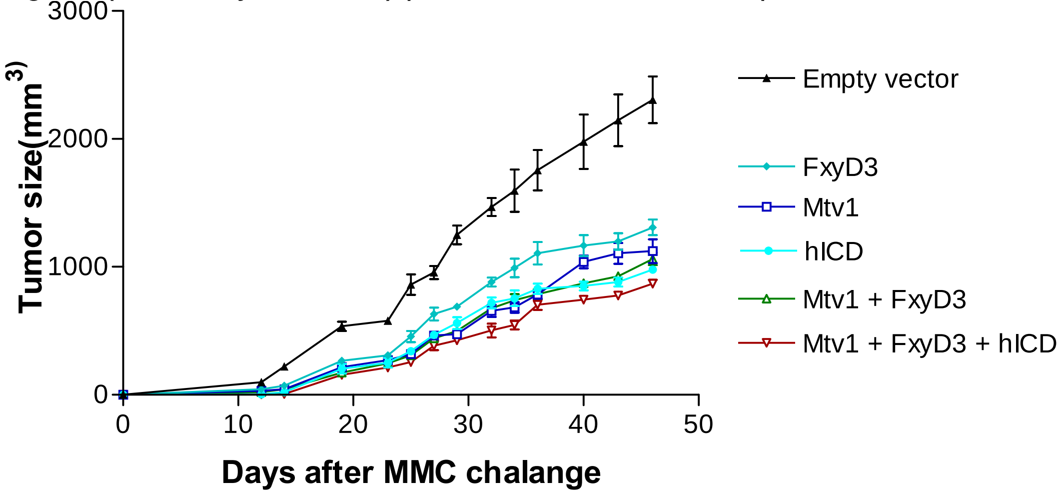

Figure 8. M ulti-antigen vaccines worked better than sin gle antigen v accines. Six-eight weeks old female neu-tg mice were vaccinated with either a single plasmid DNA (FxyD3, Mtv1, hICD, or empty vector) or combination of plasmid DNAs (Mtv1+FxyD3 or Mtv1+FxyD3+hICD) on days -42, -28, and -14. One million live MMC cells were given subcutaneously on day 0.

3.B. Vaccination with tumor rejection antigens inhibits the growth of **spontaneous tumor**.

3.B.1.Vaccination with Mtv1 and Cep290 single tumor antigen vaccine results in prolonged tumor-free and overall survival. MMTV-neu transgenic mice develop spontaneous tumors around 5-7 months of age. We tested whether the plasmid DNA vaccine encoding tumor rejection antigens can prevent the development of spontaneous tumor and prolong tumor-free and overall survival in these mice. Vaccines were administered to twelve week-old mice intra-dermally every two weeks for 3 immunizations and then monthly for 2 more immunizations. Tumor growth was measured by venier caliper. As shown in Fig. 9A and B, vaccination with Mtv1 and Cep290 resulted in significantly prolonged tumor-free survival and overall survival. Vaccination with FxyD3 did not show any protection effect. Vaccination with hICD increased overall survival but not tumor-free survival.

A B

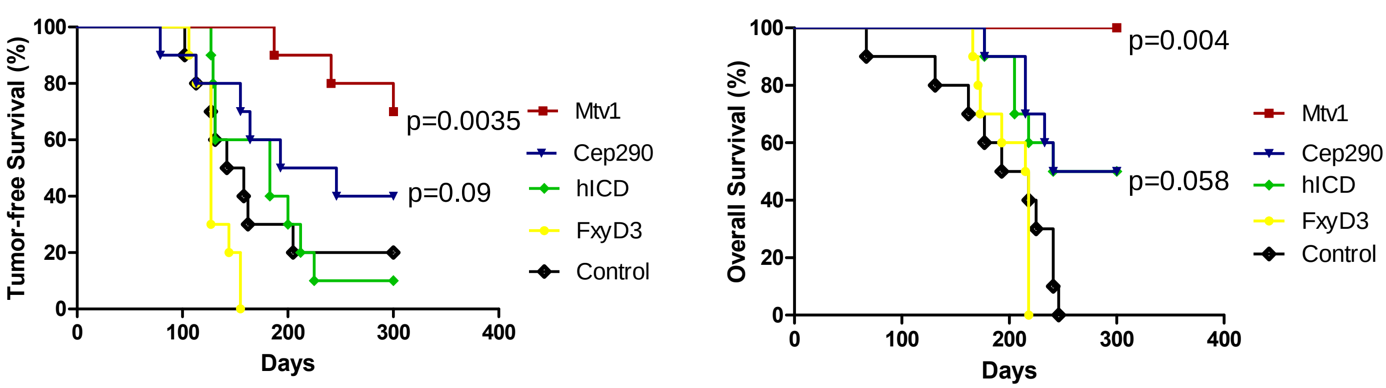

Figure 9. Va ccination using plasmid DNA e ncoding tumor rejection antigens enhanced tumor-free survival and overall survival in neu transgenic mice. Twelve weeks old neu transgenic mice (n=10 per group) received a total of 5 DNA vaccines (first three are 2 weeks a part, then monthly apart). Each vaccine consists of 50ug DNA. CFA/IFA was given as adjuvant.

3.B.2. Vaccination with a multiple antigen DNA vaccine results in prolonged tumor-free and overall survival.

We tested the combination of three antigens, Mtv1+Cep290+FxyD3 in preventing mice from developing spontaneous tumors. Young MMTV-neu transgenic mice received 5 DNA vaccines (first 3 two weeks apart the next two are 1 month apart). 60% of vaccinated mice remained tumor free at 1 year of age, as compared to 20% of controls (p=0.01, Fig. 10A). The overall survival of the mice was also significantly prolonged (p=0.01, Fig. 10B). This multi-Ag vaccine study was initiated before we knew the results of the single antigen vaccine in spontaneous tumor setting. The 3 antigens were chosen based on their protection effects in implanted tumors. Based on the finding that FxyD3 didn't protect development of spontaneous tumor, future experiment should further optimize the vaccine combination by replacing FxyD3 with another plasmid vaccine such as hICD.

A B

p=0.0125 p=0.0125

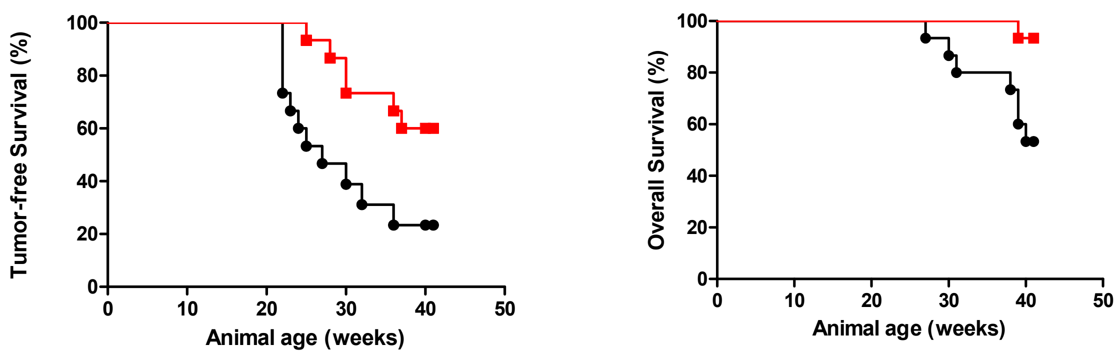

Figure 10. Vaccination targeting three tumor rejection antigens significantly prolonged the tumor-free and overall surv ival in neu transgenic mice. Twelve weeks old neu transgenic mice were randomly assigned to 2 treatment groups, multi-Ag DNA vaccine (●, black line) or control group (■, red line). The mice received a total of 5 DNA vaccines (first three are 2 weeks a part, then monthly apart). Each vaccine consists of 50ug DNA. CFA/IFA was given as adjuvant. Shown are tumor-free survival (A) and overall survival (B) in each group (n=15 per group).

In summary, we have identified a panel of tumor rejection antigens using mouse tumor rejection models. More than half of the mouse tumor rejection antigens (6/10) have immunogenic human homologues. Vaccination using plasmid DNA encoding the tumor rejection antigens significantly inhibits the development of both implanted and spontaneous tumors in MMTV-neu transgenic mice.

Reportable Outcomes

A. Publication:

1. Knutson KL, Dang Y, Lu H, et al. IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in neu-transgenic mice. J Immunol 2006; 177: 84-91.
2. Lu H, Knutson KL, Gad E, Disis ML. The tumor antigen repertoire identified in tumor-bearing Neu transgenic mice predicts human tumor antigens. Cancer Res 2006; 66: 9754-61.
3. Lu H, Gad E, Chang A, Larson E, Disis ML. Identification of tumor rejection antigens using mouse tumor rejection models. manuscript in preparation.

B. Presentations:

1. Lu H, Gad E, Chang A, Seymour K, and Disis ML. The identification of tumor rejection antigens in murine models that are associated with human homologues. Poster presentation at AACR, 2006

2. Lu H, Chang A, Larson E, Gad E, and Disis ML. Identification of an immunological signature of tumor rejection in the neu transgenic mouse. Oral presentation at AACR, 2007

3. Lu H, Chang A, Gad E, Larson E, Park E, La S, Disis ML. Vaccination targeting antigens identified in tumor rejection mice but not antigens identified in tumor bearing mice has tumor protective effect. Poster presentation at DOD Era of Hope meeting, 2008

4. Disis ML, Gad E, Cecil D, Park K, Lai V, Lubet R, and Lu H. Preventing the development of breast cancer by immunizing with multi-antigen vaccines targeting proteins associated with oncogenesis. To be presented at San Antonio Breast Cancer Conference, 2009

Conclusions:

Mouse tumor rejection models are very helpful in identifying tumor antigens that are potentially of therapeutic value for humans. We have identified a panel of mouse tumor rejection antigens. More than half of the mouse tumor rejection antigens have immunogenic human homologues. Vaccination using plasmid DNA encoding the tumor rejection antigens can inhibit tumor growth in mice.

IL-2 Immunotoxin Therapy Modulates Tumor-Associated Regulatory T Cells and Leads to Lasting Immune-Mediated Rejection of Breast Cancers in *neu*-Transgenic Mice[1]

Keith L. Knutson,[2] Yushe Dang, Hailing Lu, Jason Lukas, Bond Almand, Ekram Gad, Ehizoje Azeke, and Mary L. Disis

Studies in cancer patients have suggested that breast tumors recruit regulatory T cells (Tregs) into the tumor microenvironment. The extent to which local Tregs suppress antitumor immunity in breast cancer is unknown. We questioned whether inhibiting systemic Tregs with an IL-2 immunotoxin in a model of *neu*-mediated breast cancer, the *neu*-transgenic mouse, could impact disease progression and survival. As in human breast cancer, cancers that develop in these mice attract Tregs into the tumor microenvironment to levels of ~10–25% of the total $CD4^+$ T cells. To examine the role of Tregs in blocking immune-mediated rejection of tumor, we depleted $CD4^+CD25^+$ T cells with an IL-2 immunotoxin. The treatment depleted Tregs without concomitant lymphopenia and markedly inhibited tumor growth. Depletion of Tregs resulted in a persistent antitumor response that was maintained over a month after the last treatment. The clinical response was immune-mediated because adoptive transfer of Tregs led to a complete abrogation of the therapeutic effects of immunotoxin treatment. Further, Treg down-modulation was accompanied by increased Ag-specific immunity against the *neu* protein, a self Ag. These results suggest that Tregs play a major role in preventing an effective endogenous immune response against breast cancer and that depletion of Tregs, without any additional immunotherapy, may mediate a significant antitumor response. *The Journal of Immunology*, **2006, 177: 84–91.**

Based on several observations in recent years, it has been suggested that breast cancer is a naturally immunogenic tumor (1–9). This naturally induced immunity to breast cancer, however, is not sufficient to block tumor growth. Several potential immune evasion mechanisms have been identified including loss of Ag processing and presentation, production of immunosuppressive cytokines, and recruitment of immunosuppressive cells into the microenvironment, such as plasmacytoid dendritic cells, and regulatory T cells (Tregs)[3] (10, 11).

An intense focus on Tregs in recent years has aided in our understanding of how these specialized T cells are able to dampen immunity (12). In breast tumors, it has been observed that Tregs are associated with the tumor microenvironment (13, 14). However, it is unclear whether this association is pathologic. Unlike other tumor types such as fibrosarcoma, the numbers of Tregs that associate with breast tumors are fewer, and in fact represent only a small minority of the total $CD4^+$ T cells (13–16). Thus, methods for selectively depleting Tregs are required to discern their role in tumor immune evasion.

In this study, our goal was to further understand the potential role of breast cancer-associated Tregs in tumor growth and pathogenesis. We used the rat *neu*-transgenic mouse to discern that role (17). In this model, Tregs infiltrate into breast cancer lesions to levels similar to those observed in human breast cancer as a small subset of the total $CD4^+$ T lymphocytes. To selectively deplete the Tregs systemically, we used an IL-2 immunotoxin fusion protein, Denileukin Diftitox (18, 19). This fusion protein consists of the enzymatically active fragment A of diphtheria toxin, the membrane-translocating portion of diphtheria toxin fragment B, and human IL-2. Studies have shown that the immunotoxin binds specifically to IL-2R in vitro and is rapidly internalized via receptor mediated endocytosis (18, 19). Immunotoxin treatment resulted in the selective depletion of Tregs without eliciting additional cytopenia. Furthermore, simple inhibition of Tregs without any additional immunomodulation had a profound effect in inhibiting tumor growth and stimulated an increase in tumor-specific immunity.

Materials and Methods

Animals

BALB/c and *neu*-transgenic mice were obtained from The Jackson Laboratory (17). The *neu*-transgenic mice harbor the nonmutated, nonactivated rat *neu* proto-oncogene under control of the mouse mammary tumor virus promoter. In these studies, only female mice, 8–12 wk old, were used for experimentation. Animal care and use was in accordance with institutional guidelines.

Reagents

FCS was obtained from Gemini Bioproducts. RPMI 1640, PBS, penicillin-streptomycin, and L-glutamine were obtained from Invitrogen Life Technologies. Fluorochrome-conjugated Abs targeting CD3, CD4, CD25, CD62L, CD69, purified and biotinylated anti-IFN-γ, and purified glucocorticoid-induced TNF-related receptor (GITR) Ab were obtained from BD Pharmingen. The IL-2 immunotoxin, Denileukin Diftitox, was a gift from D. Woo and K. Brady of Ligand Pharmaceuticals. Denileukin Diftitox is very rapidly cleared and in humans and rodents,

Department of Immunology, Mayo Clinic College of Medicine, Rochester, MN 55905; and Center for Translational Medicine in Women's Health, Tumor Vaccine Group, University of Washington, Seattle, WA 98109

Received for publication January 27, 2006. Accepted for publication April 10, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Cancer Institute Grants R01-CA85374 (to M.L.D.), K24-CA85218 (to M.L.D.), R01 CA-113861 (to K.L.K.), and K01-CA100764 (to K.L.K.).

[2] Address correspondence and reprint requests to Dr. Keith L. Knutson, Mayo Clinic College of Medicine, 342C Guggenheim, 200 First Street Southwest, Mayo Clinic, Rochester, MN 55905. E-mail address: knutson.keith@mayo.edu

[3] Abbreviations used in this paper: Treg, regulatory T cell; GITR, glucocorticoid-induced TNF-related receptor; MMC, murine mammary carcinoma; TIL, tumor-infiltrating lymphocyte.

0022-1767/06/$02.00

the drug has a half-life of <2 h in the clearance phase (Ligand Pharmaceuticals, unpublished observation and drug package insert). [^{3}H]Thymidine was purchased from PerkinElmer. The $CD4^+CD25^+$ (Treg) T cell purification kit was purchased from Miltenyi Biotec. Alkaline phosphatase-conjugated goat anti-mouse Ig and NBT-chloride/5-bromo-4-chloro-3-indolyl-phosphate color development solution were obtained from Invitrogen Life Technologies. In general, all other reagents were purchased from Sigma-Aldrich. Foxp3 and β-actin primers and TaqMan Universal PCR Master Mix were obtained from Applied Biosystems, and Foxp3 Ab was obtained from eBioscience. Monoclonal mouse anti-CD25 Ab was produced using the PC 61.5.3 hybridoma (American Type Culture Collection (ATCC)). A first-strand cDNA synthesis kit was purchased from Novagen. The H-2Dq/$RNEU_{420-429}$ (H-2D(q)PDSLRDLSVF) tetramer was obtained from the National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA).

Cell lines

The mouse mammary carcinoma (MMC) cell line was established from a spontaneous tumor harvested from the *neu*-transgenic mice as previously described (20). MMC cells were grown and maintained in RPMI 1640 supplemented with 20% FCS as well as penicillin/streptomycin and L-glutamine.

T cell enrichment

Tumor-infiltrating lymphocytes (TIL) were harvested by mincing the tumor and screening. The TIL were then isolated from tumor cells by centrifugation of the cell suspension on a discontinuous Ficoll gradient consisting of a lower 100% layer and an upper 75% layer. The cells were gently layered onto the top of the gradient followed by centrifugation at $280 \times g$ for 30–45 min at 4°C followed by two washes in HBSS. The TIL were collected at the top of the 75% layer and the tumor cells at the top of the 100% layer. The cells were then stained for flow cytometric analysis as described below. For isolation of $CD4^+CD25^+$, the Miltenyi Biotec mouse Treg purification kit was used in conjunction with an AutoMacs machine (Miltenyi Biotec). The resulting $CD4^+CD25^+$ T cell population was consistently >95% pure as assessed by flow cytometry. Purified $CD4^+CD25^+$ T cells were then used in MLR assays as described below or reinfused into tumor-bearing animals.

Tumor growth in vitro and in vivo

For tumor studies, MMC cells, which are syngeneic with the *neu*-transgenic mouse, were used. For in vitro experiments, 1.0×10^5 MMC were plated in 6- or 96-well plates with medium alone or with varying concentrations of Denileukin Diftitox. For evaluation by flow cytometry, the wells were harvested with a NaCl solution (0.8%) with 2 mM EDTA. Proliferation analysis was done as previously described (21). For in vivo tumor growth, MMC cells were harvested using 2 mM EDTA in PBS and washed before injection. Mice were inoculated with 5×10^6 MMC cells s.c. on the mid-dorsum with a 23-gauge needle, which is a dose of tumor cells that results in the development of tumors in 100% of *neu*-transgenic mice. Tumors were measured every other day with vernier calipers and tumor volume was calculated as the product of length $\times$ width $\times$ height $\times$ 0.5236. In vivo data are presented as mean $\pm$ SEM. The numbers of mice used in each experiment are described in *Results*. For in vivo studies, statistical significance ($p < 0.05$) was determined using Student's t test by comparing the means of different treatment groups (GraphPad InStat for Windows 95/NT; GraphPad). Mice were treated every 2–3 days with tail vein dosing of either 100 μl of PBS as control or 100 μl of PBS containing varying concentration of the immunotoxin. Mice received either three or six doses of immunotoxin which is specifically stated in the figure legends. In one experiment, a depleting anti-CD25 Ab was used in parallel with the IL-2 immunotoxin for in vivo therapy. In this experiment, mice were treated as previously described with a single 1-mg dose of anti-CD25 Ab given 2 days before tumor cell injection (22).

Flow cytometry

Cell surface and intracellular marker analysis of splenocytes and TILs was done as previously described (20). Samples were run on a FACS Scan II and analyzed using CellQuest software (BD Biosciences). Foxp3-PE intracellular staining was done according to the manufacturer's intracellular staining protocol. For the tetramer experiments, mice were treated with Denileukin Diftitox followed by injection of 5×10^6 irradiated MMC tumor cells 2 days later (>10 drug half-lives). For the Foxp3 intracellular staining, values were considered significantly lower if they were below the mean and 3 SDs of the control mice value. Means were calculated from appropriate quadrant values and statistical analysis of data was done using Graphpad InStat as described above.

Proliferation assays

In vitro tumor cell and T cell proliferation were examined using a tritiated thymidine incorporation assay in 96-well plates as previously described (23). In brief, for the T cell proliferation assays, the cells were exposed for 5 days to control or experimental compounds or irradiated allogeneic stimulator cells. Allogeneic stimulators were derived from BALB/c mice spleens and irradiated to 3300 rad before use in the MLR. The stimulator cells (1×10^5) were mixed with splenocytes derived from the *neu*-transgenic mice at a 1:1 ratio. $CD4^+CD25^+$ T cells, derived from the *neu*-transgenic mice, were added to a final concentration of either 1×10^4 or 1×10^5 cells. On day 5, 50 μl of medium containing 10 μCi [^{3}H]thymidine was added per well for 8 h followed by harvesting the cells and measuring the uptake of the [^{3}H]thymidine into the DNA of the cells. Data are expressed as the mean percentage of control uptake or as a stimulation index calculated as the ratio of the mean value of the experimental wells over the mean value of the control wells.

Foxp3 mRNA analysis

Normalized PCR analysis of lymphocyte Foxp3 mRNA was done similar to what has been previously described except substituting β-actin as the normalization gene (24, 25). Total RNA was extracted from TIL cells using an RNAqueous-4PCR kit (Ambion). The cDNA synthesis was conducted using a first-strand cDNA synthesis kit. Quantitative real-time PCR was performed on a Prism 7900 sequence detection system using a primer mix for either Foxp3 or β-actin. The reaction mixtures (20 μl of total volume) contained 10 μl of TaqMan Universal PCR Master Mix, 9 μl of diluted cDNA, along with 1 μl of either primer mix. The reactions were amplified as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Relative Foxp3 mRNA expression in immunotoxin-untreated and treated mice was determined by normalizing to that of β-actin expression in each sample. The expression in immunotoxin-treated mice is presented as fold-change in mRNA values using untreated mice as a reference sample.

ELISA detection of tumor Ag-specific Abs

Serum samples were taken before and after the mice received immunotoxin or control PBS treatment. The sera were used to measure Ab response to a panel of eight SEREX-identified mouse tumor Ags (C3, Eprs, Krt2–8, Lass2, Rock1, Srpk1, Swap70, Yb1) using crude lysate ELISA. XLOLR bacteria were transformed with plasmid encoding the gene of interest or plasmids with no inserts (reference lysate) were grown in Luria-Bertani medium. Protein expression was induced with 2 mM isopropyl β-D-thiogalactoside. After overnight culture at 37°C, the bacteria were spun-down and resuspended in PBS with protease inhibitor (Roche). Cells were then disrupted by freeze-thawing and vortexing. Protein concentration was determined by BCA protein assay kit (Pierce). Bacterial lysate was frozen at −70°C until use. Ninety-six-well Immulon 4HBX microtiter plates (Dynex Technologies) were coated with XLOLR bacteria lysate (50 μg/ml diluted in carbonate buffer). Plates were coated overnight at 4°C using lysate from bacteria that express the protein or reference lysate. After blocking with 100 μl/well of PBS/1% BSA at room temperature on a rocker for 1 h, plates were washed four times with PBS/0.5% Tween 20. After washing, 50 μl of 1/100 diluted mouse sera was added to each well and incubated at room temperature on a rocker for 2 h. After serum incubation, plates were washed four times with PBS/Tween 20 and goat anti-human IgG-HRP conjugate (Zymed Laboratories; 1/5000 diluted) was added and incubated for 1 h at room temperature on a rocker. After a final PBS/Tween 20 wash, TMB developing reagent (Kirkegaard & Perry Laboratories) was added (75 μl/well) and the reaction was then stopped with 75 μl/well 1 N HCl and read at an OD of 450 nm. The OD of each serum sample was calculated as the OD of the Ag lysate-coated wells minus the OD of reference lysate-coated wells. Statistical analysis was performed as described above.

Results

The numbers of $CD4^+CD25^+Foxp3^+$ T cells are markedly increased in the breast tumors of neu*-transgenic mice as compared with the peripheral circulation*

In mice, GITR, CD62L, CD69, and Foxp3 have been shown to identify Treg populations, distinguishing them from activated

CD4$^+$CD25$^+$ (26, 27). Using a combination of these markers as well as CD25 to define Treg phenotype, we examined the baseline levels of Tregs in the spleen from non-tumor-bearing *neu*-transgenic mice (Figs. 1, *A–E*). CD4 and CD25 distinguished a population that constituted 10.4 ± 1% of spleen CD4 T cells. All four markers, CD62L, CD69, GITR, and Foxp3, identified a subpopulation of CD4$^+$CD25$^+$ T cells. CD62L, a molecule that mediates binding of naive T cells to endothelium, is lost on T cell activation and is expressed constitutively on the Tregs (28). CD62L and CD25 were coexpressed on 6.1 ± 1.1% of spleen-derived CD4$^+$ T cells ($n = 5$, Fig. 1, *A* and *B*). The absence of CD69, an early marker of T cell activation, distinguishes between CD25$^+$ Tregs and activated effector T cells. The CD25$^+$CD69$^-$ T cells, similar to that observed using CD62L as a marker, were 6.1 ± 0.6% of the spleen-derived CD4$^+$ T cells, respectively (Fig. 1, *A* and *C*). GITR also distinguished a population of similar size (5.8 ± 0.9%) of CD4 T cells compared with CD62L, in the spleen ($n = 3$, Fig. 1, *A* and *D*). Recently, a mAb has become available to demonstrate protein expression of Foxp3, a fairly specific marker for thymic-derived Tregs. Intracellular staining with this Ab revealed that 6.9 ± 0.2% ($n = 5$, Fig. 1, *A* and *E*) of the splenic CD4$^+$ T cells are Foxp3$^+$ which is consistent with the findings using the other markers. Overall, Foxp3 stained 80 ± 2% ($n = 5$) of the CD4$^+$CD25$^+$ T cells suggesting that the vast majority of, but not all, circulating CD4$^+$CD25$^+$ T cells are thymic-derived Tregs (data not shown).

Purified splenic CD4$^+$CD25$^+$ T cells from the *neu*-transgenic mouse could block T cell responses in a MLR assay in a dose-dependent manner (Fig. 1*F*). At a 1:10 Treg:effector T cell ratio, the proliferative response in a MLR was reduced to 67 ± 10% (mean ± SEM, $n = 4$, $p = 0.07$) of the control response, whereas

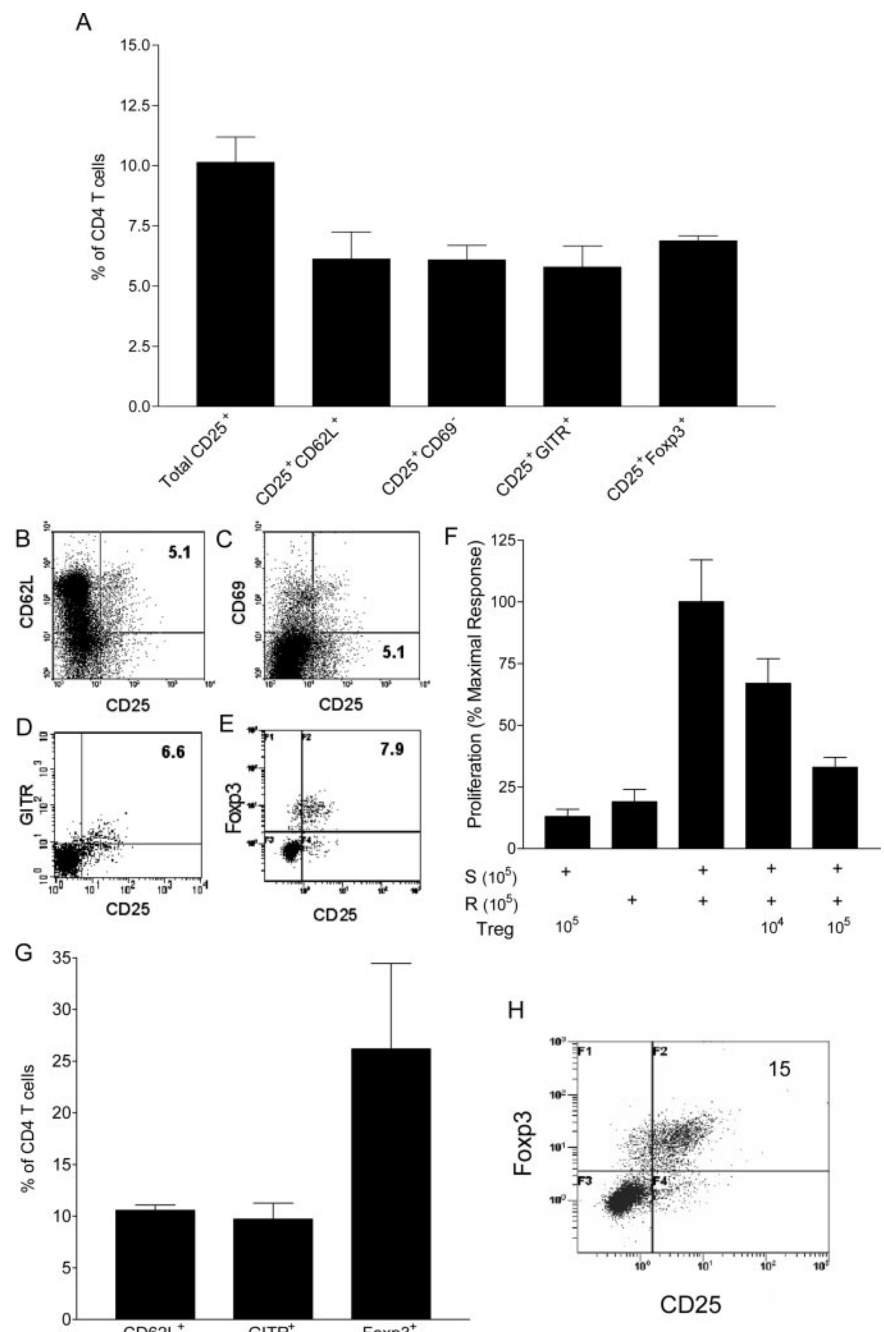

FIGURE 1. Numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells are markedly increased in the breast tumors of *neu*-transgenic mice as compared with the peripheral circulation. *A*, Mean numbers of CD4$^+$CD25$^+$ (Total CD25$^+$), CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$CD69$^-$ CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells present in spleen. The data (mean ± SEM) are calculated from 3 to 10 mice each using at least 50,000 gated CD4$^+$ T cells. *B–D*, Representative dot plots for CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$CD69$^-$, CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells, respectively. The values shown represent the percentage that the quadrant represents of the total CD4$^+$ T lymphocytes. *F,* Results of a proliferation assay with values expressed as a mean (±SEM) percentage of the highest proliferation response (S, stimulators; R, responders). Values are representative of three determinations. *G*, The mean numbers (±SEM) of CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells present in the TIL. The data are calculated from 4 to 10 mice each using at least 50,000 gated CD4$^+$ T cells. *H*, The distribution of Foxp3 among the CD4$^+$ T cell population in the tumor bed. The number shown in the *upper right quadrant* is the percentage of CD4$^+$ T cells that stained positive for both CD25 and Foxp3 in this example.

at a 1:1 ratio, the proliferative activity was reduced to 33 ± 4% ($n = 4$, $p = 0.004$) of control response.

An analysis of the TIL revealed that Tregs concentrate in the tumor tissue (Fig. 1, *G* and *H*). Tregs, as measured by CD62L$^+$ or GITR$^+$ constituted 10.6 ± 0.5% ($n = 8$, $p < 0.0001$ compared with spleen) and 9.7 ± 1.6% ($n = 9$, $p = 0.016$) of the total CD4$^+$ T cells infiltrating the tumor, respectively. In contrast, Foxp3 (along with CD25) stained 26.2 ± 8% ($n = 5$) of the infiltrating CD4$^+$ T cells suggesting that CD62L and GITR do not represent the entire Treg population or whose levels modulated during activation in the tumor microenvironment. In contrast to studies in humans, we found that levels of Treg markers were not elevated, but slightly decreased, in the periphery following tumor development (e.g., control spleen GITR$^+$ T cells, 6.1 ± 0.6%, $n = 5$; tumor spleen GITR$^+$ T cells, 4.0 ± 0.4, $n = 3$, $p = 0.02$).

CD4$^+$CD25$^+$Foxp3$^+$ T cells can be systemically depleted with an IL-2 immunotoxin without causing profound lymphopenia

In the interest of modulating immunosuppressive T cells, we examined whether CD4$^+$CD25$^+$GITR$^+$ T cells, CD4$^+$CD25$^+$CD62L$^+$ T cells, or CD4$^+$CD25$^+$Foxp3$^+$ T cells could be depleted systemically using the IL-2 immunotoxin, DAB$_{389}$IL-2. Fig. 2*A* shows that treatment with the immunotoxin can reduce the number of CD4$^+$CD25$^+$GITR$^+$ T cells in the spleen. The effects were dose dependent and observed above levels of a 1-μg dose. In animals treated with 5 μg (2.0 ± 0.4, $n = 3$, $p = 0.008$), the levels of Tregs in the spleen were significantly lower than control (PBS-treated) animals (5.8 ± 0.86, $n = 3$). As shown in Fig. 2*B*, four of six mice demonstrated reduced numbers of Foxp3$^+$ Tregs compared with control levels. During treatment, there was no change in the absolute number of CD4$^+$ and CD8$^+$ T cells in any of the groups. As shown in Fig. 2*C*, at the highest dose of immunotoxin, the relative number of CD4 T cells was 68 ± 4% (mean ±SEM, $n = 3$, $p > 0.05$ vs control) of the total spleen cells. This was not significantly different from the control level of 72 ± 0.3%. Similarly, the numbers of CD8$^+$ T cells was also not significantly altered by systemic treatment with immunotoxin. The relative number of splenic CD8$^+$ T cells was 28 ± 2% ($n = 3$) for the 5-μg dose of immunotoxin and was 24 ± 1% ($n = 3$) for the control group ($p > 0.05$).

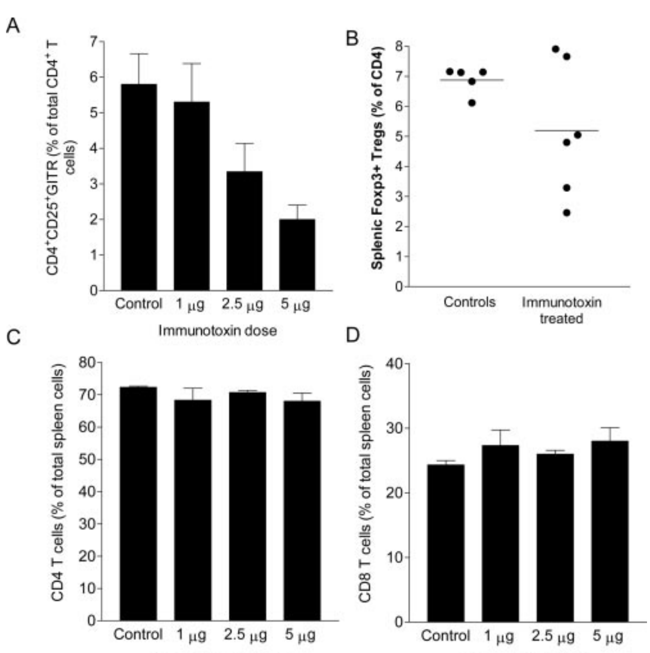

FIGURE 2. Tregs can be systemically depleted without causing profound lymphopenia. *A*, The levels of CD4$^+$CD25$^+$GITR$^+$ T cells in the spleen after systemic treatment with varying doses (total of six doses of 5, 2.5, or 1 μg every 2–3 days), shown on the *x*-axis, of immunotoxin or control medium. *B*, Levels of CD4$^+$CD25$^+$Foxp3$^+$ T cells in immunotoxin-treated mice, given 5 μg of immunotoxin every 2–3 days for a total of six doses. The levels in *A* and *B* are expressed as a percentage of the total CD4$^+$ T cells. *C* and *D*, The levels of splenic CD4$^+$ and CD8$^+$ T cells, respectively, expressed as the percent of total splenic-derived cells. Each bar represents the mean and SEM of three replicates at each dose.

Mice undergo immune-mediated tumor rejection following IL-2 immunotoxin therapy and demonstrate persistent reductions in tumor-associated Foxp3$^+$ T cells

Treatment with immunotoxin was initiated on the day after tumor challenge (i.e., day 1) in *neu*-transgenic mice before tumor development. On day 40, the mean (±SEM) tumor size in animals treated with the 1- and 5-μg dose was 666 ± 94 mm^3 ($n = 6$, $p = 0.006$ compared with control day 40) and 310 ± 136 mm^3 ($n = 5$, $p = 0.0007$), respectively (Fig. 3*A*). The mean tumor size of the control mice on day 40 was 1275 ± 156 mm^3 ($n = 6$). On day 51, tumors in the mice treated with the 5-μg dose were stable and did not differ in size compared with day 40, despite the fact that the immunotoxin treatment had ended over 30 days before that measurement ($p = 0.5$). Although the tumors in the mice treated with the 1-μg dose did continue to grow, the rate was markedly slower than control animals and on day 51 the mean tumor size was significantly smaller than control (1732 ± 295 vs 3148 ± 360, $p = 0.007$). We evaluated for Foxp3$^+$ T cell mRNA signal in TILs within these tumors using PCR which demonstrated that Tregs were reduced in the tumor microenvironment in the immunotoxin-treated animals (Fig. 3*B*, $p < 0.03$).

To demonstrate that the effects of the immunotoxin were immunologically mediated, a subsequent experiment was performed to replace Tregs that were removed with immunotoxin. Ten days after groups of tumor-challenged animals were treated with immunotoxin and had demonstrated significant persistent tumor inhibition, we replaced the Treg population either by infusion of unfractionated splenocytes or infusion of CD4$^+$CD25$^+$ purified T cells. As shown, the Treg infusion led to a complete abrogation of the effects of immunotoxin (Fig. 3*C*). By day 33, the tumors in the animals treated with immunotoxin were significantly smaller than control (181 ± 29 mm^3, $p < 0.0001$). Tumors in the group subsequently infused with purified CD4$^+$CD25$^+$ T cells (922 ± 219 mm^3, $n = 5$, $p = 0.5$) were the same size as tumors in untreated control animals (915 ± 245 mm^3, $n = 6$). Splenocytes, also a source of Tregs, partially abrogated the antitumor effect of the immunotoxin when adoptively transferred into immunotoxin-treated animals (436 ± 57 mm^3, $n = 6$).

MMC tumor cells are not directly sensitive to IL-2 immunotoxin

We next considered the possibility that IL-2 immunotoxin treatment may have a direct effect on tumor either by coating the surface for immune activation or by internalizing it to inhibit cell proliferation. As shown in Fig. 4*A*, the immunotoxin had no impact on tumor cell growth as assessed by an estimate of proliferation. At the highest in vitro dose examined, the proliferative stimulation index was 1.3 ± 0.2 (mean SI ± SEM, $n = 3$), which was not significantly different from medium control (1.0 ± 0.3) or protein (500 ng/ml OVA, oval, 1.0 ± 0.1) control ($p > 0.05$). Further, the tumors themselves do not express CD25 on the cell surface, further explaining the insensitivity to the drug (Fig. 4*B*).

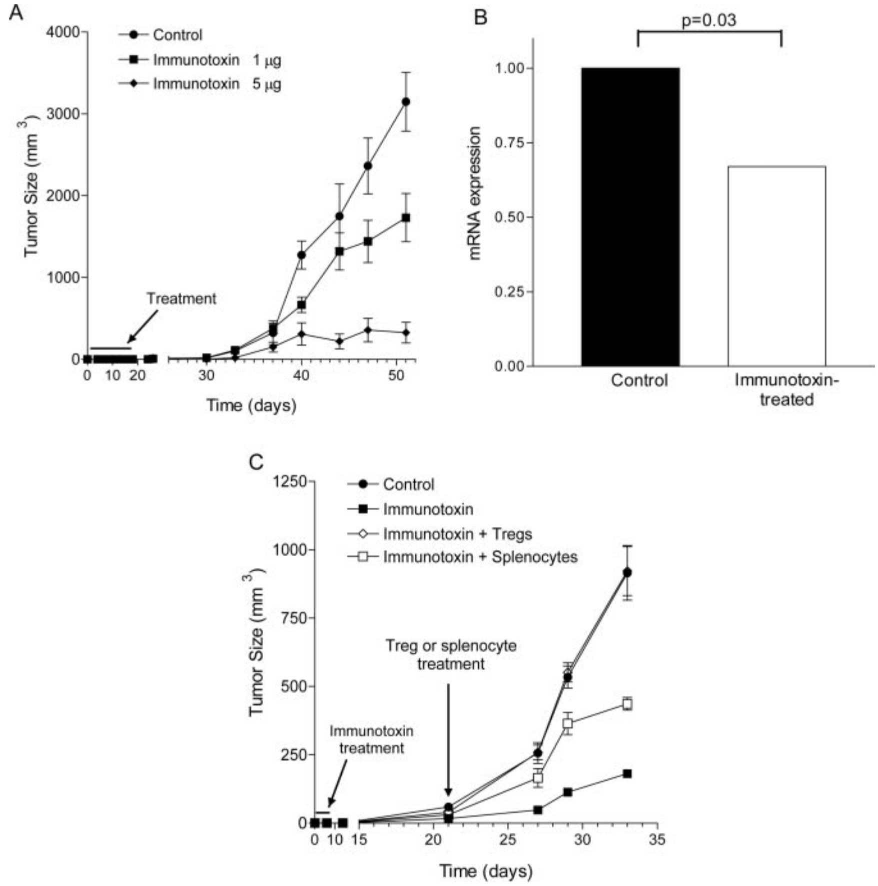

FIGURE 3. Mice undergo immune-mediated tumor rejection following IL-2 immunotoxin therapy and demonstrate persistent reductions in tumor-associated Foxp3$^+$ T cells. *A*, The line spanning the first 17 days of the time course (arrow) represents the immunotoxin (■, 1 μg; ♦, 5 μg) or PBS (control, ●) treatment time points. Mice were given six doses (1 or 5 μg/dose) of IL-2 immunotoxin, one dose given every 2–3 days. Each data point represents the mean tumor size ($\pm$SEM) of five to six mice given in cubic millimeters. Another experiment with six mice yielded similar results. *B*, Levels of intratumoral Foxp3 mRNA (calculated from five to six mice per group) in mouse tumors treated with 5 μg of IL-2 immunotoxin three times every 2–3 days. *C*, The line spanning the first 4 days of the time course (arrow) represents the immunotoxin or PBS (control, ●) treatment time points. Mice were treated with three doses of 5 μg of IL-2 immunotoxin given every 2–3 days. At day 21, a group of immunotoxin-treated mice received a CD4$^+$CD25$^+$ T cells (◇), splenocyte (□) or PBS infusion (■). Each data point represents the mean tumor size ($\pm$SEM) of five to six mice given in cubic millimeters. The closed circles and open diamonds coincide on the graph.

Mice undergoing tumor rejection following Treg depletion with IL-2 immunotoxin demonstrate elevated levels of tumor Ag-specific Abs

Given that the immunotoxin is associated with the down-regulation of Treg and generates an antitumor response, we next questioned whether immunity to the tumor Ags was being induced during treatment. As shown in Fig. 5, immunotoxin treatment was associated with the development of tumor Ag-specific immune responses as assessed by Ag-specific ELISA. Blood drawn from mice ($n = 8$) before and after treatment with the immunotoxin or PBS revealed that immunotoxin-treated mice developed IgG Ab immunity to srpk1, a novel tumor Ag our group has recently identified as a natural immune target in *neu*-transgenic mice (29).

Depletion of Tregs increases the capability of the mice to overcome tolerance to tumor-associated Ag neu

The *neu*-transgenic mouse is highly tolerant to *neu* and therefore we next examined whether immunotoxin treatment elicited a T cell response against the immunodominant epitope of rat *neu*, peptide 420–429, that has been previously reported to be targeted following depletion of Tregs with anti-CD25 mAb (22, 30). As shown in Fig. 6, the depletion of Tregs with immunotoxin led to an increased capability of mice to generate *neu*-specific CD8 T cell immunity as directly assessed with tetramer analysis. Although the results suggest that IL-2 immunotoxin is better than anti-CD25 treatment, the results were not significantly different ($p > 0.05$), which may have been due to the different dosing strategies.

Discussion

Breast cancer is a naturally immunogenic tumor. Several studies have demonstrated that breast cancer lesions are extensively infiltrated with immune effector cells such as T cells, B cells, and dendritic cells (1, 2, 31–36). Investigators have also observed that a lack of T cell infiltration in breast cancer may be associated with increased invasion of local lymph nodes by disease, indicating that an active immune response can limit the growth and spread of the tumor (35). Furthermore, tumor-Ag-specific immunity can be detected in the blood and bone marrow of breast cancer patients, but not in non-cancer-bearing donors, indicating that exposure to tumor induces a tumor Ag-specific immune response (8, 37, 38). Despite the fact that patients with breast cancer can demonstrate an immune response to their tumors and that T cells can localize to the breast tumor microenvironment, cancer growth remains unchecked. Such observations suggest that there must be a number of mechanisms in the tumor microenvironment that act simultaneously to prevent tumor destruction by the immune system. Our studies suggest that Tregs concentrate naturally in the tumors of *neu*-transgenic mice at levels above that observed in the systemic circulation. Furthermore, we also show IL-2R-directed immunotoxin therapy can reduce systemic levels of CD4$^+$CD25$^+$Foxp3$^+$ Tregs and lead to an increased capability of mice at high risk for tumor development to immunologically reject their tumors for a sustained period of time, even in the absence of therapy. This increased capability of tumor rejection was accompanied by increased tumor Ag-specific immunity.

The *neu*-transgenic mouse demonstrates profound tolerance to the spontaneous breast tumors that develop late in life and the *neu*-specific T cells that can be isolated in these animals are of low avidity (39, 40). Despite the low-avidity immune response, however, the tumor-Ag-specific T cells that remain in the body can demonstrate antitumor activity suggesting that peripheral mechanisms regulate their effector function (40). Presumably, at sites

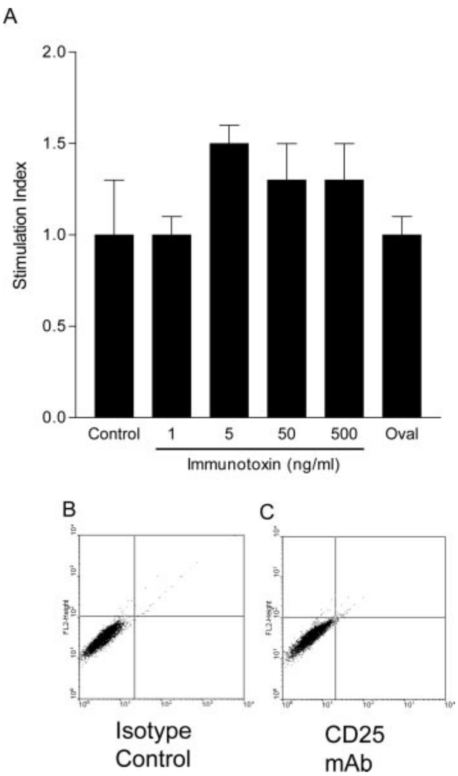

FIGURE 4. MMC tumor cells are not directly sensitive to IL-2 immunotoxin. *A*, Proliferation of tumor cells after exposure to the IL-2 immunotoxin. The results are shown as the mean stimulation index calculated from thymidine incorporation following the exposure of tumor cells to doses of PBS, immunotoxin, or OVA. Each bar represents the mean (±SEM, $n = 6$ replicates) proliferation expressed as a stimulation index calculated from the control cells. The graph is representative of two independent experiments that yielded similar results. *B* and *C*, Flow cytometric dot plots from two different tumor samples either stained with isotype control Ab (*C*) or stained for CD25. The histograms are representative of two independent experiments that yielded similar results.

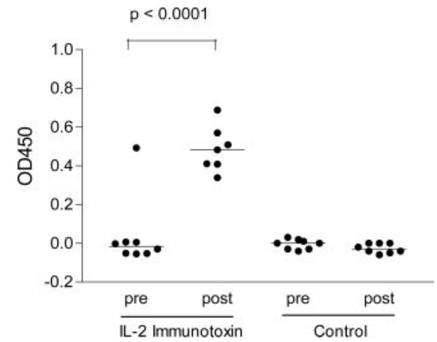

FIGURE 5. Mice undergoing tumor rejection following IL-2 immunotoxin demonstrate elevated levels of tumor Ag-specific Abs. Shown are the OD450 of ELISA specific for the tumor Ag, srpk1. The OD was calculated as the OD of Ag-coated wells minus the OD of reference lysate-coated wells. Each data symbol represents the Ab level in one mouse serum sample. The data is representative of two independent experiments.

where the Ag levels are elevated, such as in the tumor microenvironment, peripheral tolerizing mechanisms must be enhanced, relative to other tissues, to limit effector T cell function specific for self-Ags (41). Data presented here suggests that Tregs are more abundant in the tumors of these mice than in the peripheral circulation, suggesting that they are actively recruited to block effector T cell function and maintain tolerance of Ag-specific T and B cells. Similar to *neu*-transgenic mice, Tregs may be recruited into human breast tumors. Indeed, it has been observed that immunosuppressive $CD4^+CD25^+$ T cells can comprise up to 20% of the total TIL population in humans (13). In vitro studies suggest that Tregs can inhibit Ag-specific T cell activation at levels as low as 10% of the infiltrating T cell population, suggesting that the levels we observed in the tumors are sufficient to block immunity (42, 43). How breast cancers attract Tregs specifically into the microenvironment remains unknown. One recent study in ovarian cancer suggests that the attraction of $CD4^+CD25^+Foxp3^+$ T cells into the ovarian tumor microenvironment is mediated by the chemokine CCL22 which is aberrantly produced by the ovarian tumors (44). Our results suggest that the reduction of Tregs early in the course of disease development prevents the emerging tumors from using Tregs to maintain tolerance. Our finding that the small tumors that do develop in immunotoxin-treated animals have less intratumoral Treg markers (Foxp3) is consistent with this hypothesis.

Given the emerging consensus that Tregs are involved in regulating Ag-specific immune responses in several disease settings such as tumor immunity, autoimmunity, and chronic infections, there is interest in developing Treg-selective depletion strategies for research and therapeutic purposes. One of the problems with defining the role of Tregs is that commonly used strategies such as chemotherapy, anti-CD3, anti-CTLA-4, and anti-CD4 treatment, can result in severe myeloablation or immune dysregulation that could lead to significant toxicities or interfere with generation of immune responses (45–47). Lymphodepletion can suppress immune responses by a number of mechanisms including retarding dendritic cells and macrophages for extended periods, both of which would be required to activate Ag-specific T cells (48). The immunotoxin strategy presented here depleted Tregs without causing myeloablation and resulted in a tumor-specific immune response. The clinical translation of Denileukin Diftitox to deplete Tregs in cancer patients is ongoing. Vieweg and colleagues (49) reported that treatment of cancer patients with Denileukin Diftitox before vaccine therapy could substantially reduce the number of $CD4^+CD25^{high}$ Tregs without altering the levels of other CD4 T cells (memory and naive). Furthermore, this selective depletion led to an apparent enhancement of immunity after vaccination. A recent report evaluated the IL-2 immunotoxin approach as monotherapy for the treatment of advanced stage refractory melanoma (50). The use of Denileukin Diftitox resulted in depletion of $Foxp3^+$ Tregs in some patients but not in others and depletion appeared to be related to dose as we have shown here. Little clinical activity was observed in these patients, which may be due to the advanced stage and potential immunosuppression in these patients. Data presented here used Denileukin Diftitox early in the course of tumor implant, mimicking a more limited stage of disease.

It should be noted that tumors of different tissue origins may use different tolerizing mechanisms. For example, in mouse fibrosarcomas, $CD4^+CD25^+$ T cells constitute nearly 70% of the total lymphocyte (total CD4 and CD8) population and the only effector T cell required for rejection was the $CD8^+$ T cells (16). In our study, we find that $CD4^+CD25^+$ T cells constitute only a minor fraction of the infiltrating CD4 T lymphocytes and this holds true even in more advanced tumors (data not shown). Furthermore, it has been shown that $CD4^+$ Th cells are essential for tumor rejection in *neu*-transgenic mice (15). Investigators demonstrated that depletion of $CD4^+$ T cells diminished the tumor rejection response as well as tumor-specific Ab production. The reconstitution, however, of tumor-specific IgG was able to recover some antitumor

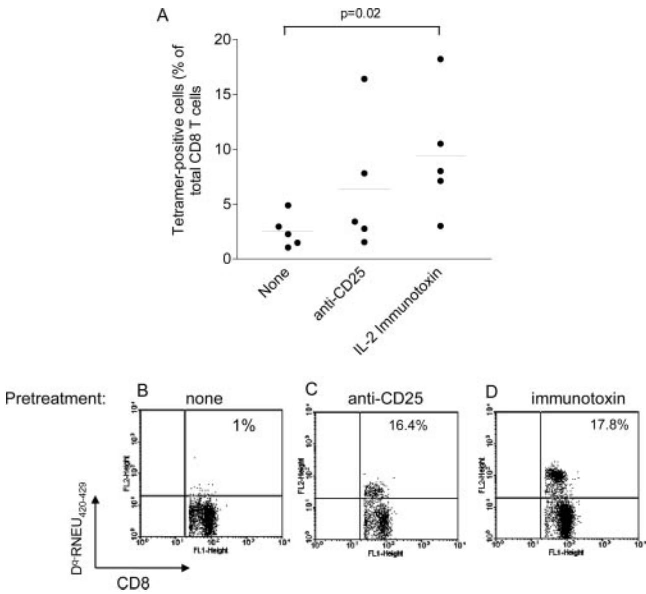

FIGURE 6. Depletion of Tregs increases the capability of the mice to overcome tolerance to tumor-associated Ag *neu. A*, The frequencies of *neu* peptide-specific CD8 T cells as assessed by tetramer analysis in mice treated with PBS, anti-CD25 mAb (1 mg mAb in a single dose), or IL-2 immunotoxin (three doses, 5 μg/dose, 2–3 days apart) 2 days before tumor cell injection. Cells were gated on CD8$^+$CD62L^{low} T cells. *B–D*, Representative dot plots.

responses indicating the important role of the CD4$^+$ T cell in enhancing the Ab responses associated with tumor rejection. Tolerization and antitumor effector mechanisms may be different in different tumors. Data presented here show that inhibition of Tregs both increases the number of tumor-specific T cells as well as allows the generation of tumor-specific Ab immunity. Whether depletion of Tregs would have such a profound effect in other models is not known.

In conclusion, our results strongly suggest that tumors that develop in *neu*-transgenic mice actively recruit Tregs to block tumor rejection and play an important role in suppressing the endogenous immune response to breast cancer. Indeed, simple inhibition of Treg function can lead to an effective antitumor response without any additional immunomodulation. Depleting Tregs in human breast cancer may stabilize disease for extended periods of time or if used in the minimal disease setting may increase the relapse-free period. Understanding the mechanisms by which breast tumors evade immunity could ultimately lead to the better design of immune-based strategies for the treatment of the disease.

Acknowledgments

We are grateful to Drs. Weiping Zou and David Woo for helpful discussions and to Marshall Behrens for expert technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Kotlan, B., P. Simsa, J. Foldi, W. H. Fridman, M. Glassy, M. McKnight, and J. L. Teillaud. 2003. Immunoglobulin repertoire of B lymphocytes infiltrating breast medullary carcinoma. *Hum. Antibodies* 12: 113–121.
2. Treilleux, I., J. Y. Blay, N. Bendriss-Vermare, I. Ray-Coquard, T. Bachelot, J. P. Guastalla, A. Bremond, S. Goddard, J. J. Pin, C. Barthelemy-Dubois, and S. Lebecque. 2004. Dendritic cell infiltration and prognosis of early stage breast cancer. *Clin. Cancer Res.* 10: 7466–7474.
3. Peoples, G. E., S. Blotnick, K. Takahashi, M. R. Freeman, M. Klagsbrun, and T. J. Eberlein. 1995. T lymphocytes that infiltrate tumors and atherosclerotic plaques produce heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor: a potential pathologic role. *Proc. Natl. Acad. Sci. USA* 92: 6547–6551.
4. Lucin, K., Z. Iternicka, and N. Jonjic. 1994. Prognostic significance of T-cell infiltrates, expression of β_2-microglobulin and HLA-DR antigens in breast carcinoma. *Pathol. Res. Pract.* 190: 1134–1140.
5. Nzula, S., J. J. Going, and D. I. Stott. 2003. Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas. *Cancer Res.* 63: 3275–3280.
6. Tsuge, T., M. Yamakawa, and M. Tsukamoto. 2000. Infiltrating dendritic/Langerhans cells in primary breast cancer. *Breast Cancer Res. Treat.* 59: 141–152.
7. Disis, M. L., E. Calenoff, G. McLaughlin, A. E. Murphy, W. Chen, B. Groner, M. Jeschke, N. Lydon, E. McGlynn, R. B. Livingston, et al. 1994. Existent T cell and antibody immunity to HER-2/*neu* protein in patients with breast cancer. *Cancer Res.* 54: 16–20.
8. Disis, M. L., K. L. Knutson, K. Schiffman, K. Rinn, and D. G. McNeel. 2000. Pre-existent immunity to the HER-2/*neu* oncogenic protein in patients with HER-2/*neu* overexpressing breast and ovarian cancer. *Breast Cancer Res. Treat.* 62: 245–252.
9. Nagorsen, D., C. Scheibenbogen, F. M. Marincola, A. Letsch, and U. Keilholz. 2003. Natural T cell immunity against cancer. *Clin. Cancer Res.* 9: 4296–4303.
10. Knutson, K. L., M. R. Bishop, K. Schiffman, and M. L. Disis. 2002. Immunotherapy for breast cancer. *Cancer Chemother. Biol. Response Modif.* 20: 351–369.
11. Knutson, K. L., K. Schiffman, K. Rinn, and M. L. Disis. 1999. Immunotherapeutic approaches for the treatment of breast cancer. *J. Mammary Gland Biol. Neoplasia* 4: 353–365.
12. Baecher-Allan, C., V. Viglietta, and D. A. Hafler. 2004. Human CD4$^+$CD25$^+$ regulatory T cells. *Semin. Immunol.* 16: 89–98.
13. Liyanage, U. K., T. T. Moore, H. G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein, P. S. Goedegebuure, and D. C. Linehan. 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol.* 169: 2756–2761.
14. Wolf, A. M., D. Wolf, M. Steurer, G. Gastl, E. Gunsilius, and B. Grubeck-Loebenstein. 2003. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin. Cancer Res.* 9: 606–612.
15. Reilly, R. T., J. P. Machiels, L. A. Emens, A. M. Ercolini, F. I. Okoye, R. Y. Lei, D. Weintraub, and E. M. Jaffee. 2000. The collaboration of both humoral and cellular HER-2/*neu*-targeted immune responses is required for the complete eradication of HER-2/*neu* transgenic mice. *Cancer Res.* 60: 3569–3576.
16. Yu, P., Y. Lee, W. Liu, T. Krausz, A. Chong, H. Schreiber, and Y. X. Fu. 2005. Intratumor depletion of CD4$^+$ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J. Exp. Med.* 201: 779–791.
17. Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* 89: 10578–10582.
18. Kreitman, R. J. 1999. Immunotoxins in cancer therapy. *Curr. Opin. Immunol.* 11: 570–578.
19. Murphy, J. R., and J. C. vanderSpek. 1995. Targeting diphtheria toxin to growth factor receptors. *Semin. Cancer Biol.* 6: 259–267.
20. Knutson, K. L., B. Almand, Y. Dang, and M. L. Disis. 2004. *neu* antigen-negative variants can be generated after *neu*-specific antibody therapy in *neu* transgenic mice. *Cancer Res.* 64: 1146–1151.
21. Knutson, K. L., and M. L. Disis. 2004. IL-12 enhances the generation of tumour antigen-specific Th1 CD4 T cells during ex vivo expansion. *Clin. Exp. Immunol.* 135: 322–329.
22. Ercolini, A. M., B. H. Ladle, E. A. Manning, L. W. Pfannenstiel, T. D. Armstrong, J. P. Machiels, J. G. Bieler, L. A. Emens, R. T. Reilly, and E. M. Jaffee. 2005. Recruitment of latent pools of high-avidity CD8$^+$ T cells to the antitumor immune response. *J. Exp. Med.* 201: 1591–1602.
23. Disis, M. L., F. M. Shiota, D. G. McNeel, and K. L. Knutson. 2003. Soluble cytokines can act as effective adjuvants in plasmid DNA vaccines targeting self tumor antigens. *Immunobiology* 207: 179–186.
24. Nishikawa, H., T. Kato, I. Tawara, H. Ikeda, K. Kuribayashi, P. M. Allen, R. D. Schreiber, L. J. Old, and H. Shiku. 2005. IFN-γ controls the generation/activation of CD4$^+$CD25$^+$ regulatory T cells in antitumor immune response. *J. Immunol.* 175: 4433–4440.
25. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
26. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, M. Mao, Y. Ding, and J. S. Bromberg. 2004. CD4$^+$CD25$^+$CD62$^+$ T-regulatory cell subset has optimal suppressive and proliferative potential. *Am. J. Transplant* 4: 65–78.
27. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4$^+$CD25$^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
28. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
29. Lu, H., K. L. Knutson, B. Stone, and M. L. Disis. 2005. Identification of tumor antigens in transgenic mice may predict immunogenic human homologues. *Proc. Am. Assoc. Cancer Res.* 46: 5121 (Abstr.).
30. Ercolini, A. M., J. P. Machiels, Y. C. Chen, J. E. Slansky, M. Giedlen, R. T. Reilly, and E. M. Jaffee. 2003. Identification and characterization of the immunodominant rat HER-2/*neu* MHC class I epitope presented by spontaneous mammary tumors from HER-2/*neu*-transgenic mice. *J. Immunol.* 170: 4273–4280.

31. Iwamoto, M., H. Shinohara, A. Miyamoto, M. Okuzawa, H. Mabuchi, T. Nohara, G. Gon, M. Toyoda, and N. Tanigawa. 2003. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int. J. Cancer* 104: 92–97.
32. Coventry, B. J., and J. Morton. 2003. CD1a-positive infiltrating-dendritic cell density and 5-year survival from human breast cancer. *Br. J. Cancer* 89: 533–538.
33. Balch, C. M., L. B. Riley, Y. J. Bae, M. A. Salmeron, C. D. Platsoucas, A. von Eschenbach, and K. Itoh. 1990. Patterns of human tumor-infiltrating lymphocytes in 120 human cancers. *Arch. Surg.* 125: 200–205.
34. Wintzer, H. O., W. Bohle, and S. von Kleist. 1991. Study of the relationship between immunohistologically demonstrated lymphocytes infiltrating human breast carcinomas and patients' survival. *J. Cancer Res. Clin. Oncol.* 117: 163–167.
35. Lucin, K., Z. Iternicka, and N. Jonjic. 1994. Prognostic significance of T-cell infiltrates, expression of β2-microglobulin and HLA-DR antigens in breast carcinoma. *Pathol. Res. Pract.* 190: 1134–1140.
36. Ben-Hur, H., O. Cohen, D. Schneider, P. Gurevich, R. Halperin, U. Bala, M. Mozes, and I. Zusman. 2002. The role of lymphocytes and macrophages in human breast tumorigenesis: an immunohistochemical and morphometric study. *Anticancer Res.* 22: 1231–1238.
37. Rentzsch, C., S. Kayser, S. Stumm, I. Watermann, S. Walter, S. Stevanovic, D. Wallwiener, and B. Guckel. 2003. Evaluation of pre-existent immunity in patients with primary breast cancer: molecular and cellular assays to quantify antigen-specific T lymphocytes in peripheral blood mononuclear cells. *Clin. Cancer Res.* 9: 4376–4386.
38. Beckhove, P., M. Feuerer, M. Dolenc, F. Schuetz, C. Choi, N. Sommerfeldt, J. Schwendemann, K. Ehlert, P. Altevogt, G. Bastert, et al. 2004. Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. *J. Clin. Invest.* 114: 67–76.
39. Kurt, R. A., R. Whitaker, A. Baher, S. Seung, and W. J. Urba. 2000. Spontaneous mammary carcinomas fail to induce an immune response in syngeneic FVBN202 *neu* transgenic mice. *Int. J. Cancer* 87: 688–694.
40. Lustgarten, J., A. L. Dominguez, and C. Cuadros. 2004. The $CD8^+$ T cell repertoire against Her-2/*neu* antigens in *neu* transgenic mice is of low avidity with antitumor activity. *Eur. J. Immunol.* 34: 752–761.
41. Nishikawa, H., T. Kato, K. Tanida, A. Hiasa, I. Tawara, H. Ikeda, Y. Ikarashi, H. Wakasugi, M. Kronenberg, T. Nakayama, et al. 2003. $CD4^+CD25^+$ T cells responding to serologically defined autoantigens suppress antitumor immune responses. *Proc. Natl. Acad. Sci. USA* 100: 10902–10906.
42. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.
43. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. *J. Exp. Med.* 193: 1303–1310.
44. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
45. Lutsiak, M. E., R. T. Semnani, R. De Pascalis, S. V. Kashmiri, J. Schlom, and H. Sabzevari. 2005. Inhibition of $CD4^+25^+$ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* 105: 2862–2868.
46. Klebanoff, C. A., H. T. Khong, P. A. Antony, D. C. Palmer, and N. P. Restifo. 2005. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends Immunol.* 26: 111–117.
47. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117: 265–277.
48. Zhang, Y., J. P. Louboutin, J. Zhu, A. J. Rivera, and S. G. Emerson. 2002. Preterminal host dendritic cells in irradiated mice prime $CD8^+$ T cell-mediated acute graft-versus-host disease. *J. Clin. Invest.* 109: 1335–1344.
49. Dannull, J., Z. Su, D. Rizzieri, B. K. Yang, D. Coleman, D. Yancey, A. Zhang, P. Dahm, N. Chao, E. Gilboa, and J. Vieweg. 2005. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J. Clin. Invest.* 115: 3623–3633.
50. Attia, P., A. V. Maker, L. R. Haworth, L. Rogers-Freezer, and S. A. Rosenberg. 2005. Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J. Immunother.* 28: 582–592.

The Tumor Antigen Repertoire Identified in Tumor-Bearing *Neu* Transgenic Mice Predicts Human Tumor Antigens

Hailing Lu, Keith L. Knutson, Ekram Gad, and Mary L. Disis

Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, Seattle, Washington

Abstract

FVB/N mice transgenic for nontransforming rat *neu* develop spontaneous breast cancers that are *neu* positive and estrogen receptor negative, mimicking premenopausal human breast cancer. These animals have been widely used as a model for immunobased therapies targeting HER-2/*neu*. In this study, we used serological analysis of recombinant cDNA expression libraries to characterize the antigenic repertoire of *neu* transgenic (*neu*-tg) mice and questioned the ability of this murine model to predict potential human tumor antigens. After screening 3 × 10^6 clones from 3 different cDNA libraries, 15 tumor antigens were identified, including cytokeratin 2-8, glutamyl-prolyl-tRNA synthetase, complement C3, galectin 8, and serine/threonine-rich protein kinase 1. Multiple proteins involved in the Rho/Rho-associated, coiled coil–containing protein kinase (Rock) signal transduction pathway were found to be immunogenic, including Rock1, Rho/Rac guanine nucleotide exchange factor 2, and schistosoma mansoni adult worm antigen preparation 70. All of the identified antigens are self-proteins that are expressed in normal tissues in addition to breast tumors and the majority of the antigens are intracellular proteins. More than half of the mouse tumor antigens have human homologues that have been reported previously as tumor antigens. Finally, the tumor-specific antibody immunity and marked immune cell infiltration that was observed in mice with spontaneous tumors were not observed in mice with transplanted tumors. Our results indicate that *neu*-tg mice bearing spontaneous tumors develop humoral immunity to their tumors similar to cancer patients and that tumor antigens identified in transgenic mouse may predict immunogenic human homologues. (Cancer Res 2006; 66(19): 9754-61)

Introduction

The development of cancer vaccines and other immunobased therapeutics has been facilitated over the last several years by the identification of a multitude of tumor antigens. In fact, to date, >2,000 cancer-related immunogenic proteins have been identified (1). It is unknown, however, which of these proteins would potentially be "tumor rejection" antigens (i.e., those proteins, when targeted by the immune system, would mediate tumor destruction). Furthermore, it is unknown what factors or characteristics make a tumor-associated proteins immunogenic. We questioned whether endogenous tumor immunity in a transgenic mouse could mimic the antigenic repertoire in human cancers and potentially provide a model for addressing some of these questions. The *neu* transgenic (*neu*-tg) mouse is a model of *neu*-mediated, estrogen receptor (ER)–negative breast cancer that has significant biologic and pathologic similarity to human breast cancer (2–4).

We used the high-throughput screening method serological analysis of recombinant cDNA expression libraries (SEREX) to identify the tumor antigen repertoire in *neu*-tg mice. Pooled sera from transgenic mice bearing spontaneous tumors were used to screen three libraries, two made from syngeneic tumor cell lines and one from testis. Approximately 3 million plaques were screened. Results showed that the majority of identified antigens were associated with immunogenic human homologues. The *neu*-tg mouse represents a useful model for tumor antigen discovery and immunotherapeutic testing, which may closely mirror human premenopausal breast cancer.

Materials and Methods

Mice and tumor cell lines. *Neu*-tg mice [strain name, FVB/N-TgN(MMTV*neu*)-202Mul] were obtained from Charles River Laboratory (Bar Harbor, ME) and bred under specific pathogen-free conditions at the University of Washington (Seattle, WA). The mice harbor nonmutated, nonactivated rat *neu* under control of the mouse mammary tumor virus (MMTV) promoter. For SEREX screening, serum samples were collected from animals bearing spontaneous tumors and control tumor-free female mice. Animal care and use was in accordance with institutional guidelines. Mouse mammary carcinoma (MMC) cells were derived from a spontaneous tumor in a *neu*-tg mouse. Antigen-negative variant (ANV) cells were derived from a rat *neu*-negative tumor that developed in a parental FVB mouse after partially rejecting an MMC implantation (5). For tumor implantation experiment, MMC cells were harvested using 2 mmol/L EDTA in PBS and washed before injection. Mice were inoculated with 1 × 10^6 MMC cells s.c. on the mid-dorsum with a 23-gauge needle. Tumors were measured every other day with Vernier calipers and tumor volume was calculated as the product of length × width × height × 0.5236. *In vivo* data are presented as mean ± SE.

RNA extraction and construction of cDNA libraries. Poly(A)$^+$ RNA was isolated from MMC and ANV cells using RNA4Aqeous and Poly(A)Purist kit from Ambion (Austin, TX). cDNA expression libraries were constructed using a ZAP Express vector from Stratagene (La Jolla, CA) following the manufacturer's instructions. The primary MMC and ANV library each contained ~1 × 10^6 recombinants and was amplified once before immunoscreening. To check the heterogeneity of the primary library, 20 plaques were randomly picked and PCR with T3 and T7 primer was done to validate the cDNA insert. The inserts ranged from 500 to 3,500 bp. A commercially available mouse testis cDNA library from Stratagene was also used for screening.

SEREX screening using murine sera. A total of 1 × 10^6 recombinant clones per library were screened with pooled sera from 10 tumor-bearing mice using the method as originally described by Sahin et al. (6) with some modifications. Briefly, 5 × 10^3 phage clones were plated with XL-Blue on NZY agar plates. After 4 hours of incubation at 37°C, isopropyl-L-thio-β-D-galactopyranoside (IPTG)–impregnated nitrocellulose membrane was

---

Note: Current address for K.L. Knutson: Department of Immunology, Mayo Clinic, Rochester, MN 55906.

Requests for reprints: Hailing Lu, Tumor Vaccine Group, University of Washington, Box 358050, Seattle, WA 98195. Phone: 206-616-8448; Fax: 206-685-3128; E-mail: hlu@u.washington.edu.

doi:10.1158/0008-5472.CAN-06-1083

overlaid on the plates to induce protein expression. Nitrocellulose membranes were removed from the plates after an overnight incubation. The membrane was first washed in TBS with 0.05% Tween 20, blocked in TBS (20 mmol/L Tris-HCl and 150 mmol/L NaCl) with bovine serum albumin (BSA), and then incubated with 1:200 diluted sera (in TBS with 1% BSA and 0.05% sodium azide) overnight at room temperature. Preliminary experiments showed that mouse sera had nondetectable levels of anti-*Escherichia coli* antibody most likely because of their controlled environment (data not shown), so no preabsorption with *E. coli* lysate was required. Instead, we did a pseudoscreening with lytic membrane to remove nonspecific binding. Alkaline phosphatase–conjugated goat anti-mouse antibody (diluted 1:2,000) was used as the secondary antibody. Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate was used for color development. Positive clones that did not react to control sera from normal mice were purified to monoclonity and converted to pBluescript phagemid by *in vivo* excision using XLOLR cells and ExAssist helper phage (Stratagene). Plasmid DNA was prepared using a FastPlasmid kit (Eppendorf, Hamburg, Germany). The nucleotide sequences of the cDNA inserts were determined using Big Dye reaction and ABI Prism automated DNA sequencer. Blast was used to search for sequence homology.

Crude lysate ELISA for the detection of tumor antigen–specific antibodies. Crude lysates of bacteria expressing the protein of interest were used as a source of the specific antigens as described by Tureci et al. (7). A newly identified phage clone was excised *in vivo* to pBluescript phagemid allowing IPTG-inducible prokaryotic expression of inserts. The XLOLR bacteria were transformed with plasmid encoding the gene of interest or plasmid with no inserts (reference lysate) and grown in Luria-Bertani medium. Protein expression was induced with 2 mmol/L IPTG. After overnight culture at 37°C, the bacteria were spun down and resuspended in 2 mL PBS with protease inhibitor (Roche, Mannheim, Germany). Protein concentration was determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Bacterial lysate was frozen at −70°C until use in ELISA or Western blot. Ninety-six-well Immulon 4HBX microtiter plates (Dynex Technologies, Inc., Chantilly, VA) were coated with bacteria lysate (50 μg/mL diluted in carbonate buffer) overnight at 4°C. After blocking with 100 μL/well of PBS/1% BSA at room temperature on a rocker for 1 hour, plates were washed four times with PBS/0.5% Tween 20 and then 50 μL mouse sera (diluted 1:100) were added to each well and incubated at room temperature on a rocker for 2 hours. After serum incubation, plates were washed four times with PBS/Tween 20 and goat anti-mouse IgG-horseradish peroxidase conjugate (diluted 1:5,000; Zymed, San Francisco, CA) was added and incubated for 1 hour at room temperature on rocker. Following final PBS/Tween 20 wash, TMB developing reagent (Kirkegaard and Perry, Gaithersburg, MD) was added (75 μL/well) and reaction was then stopped with 75 μL/well 1 N HCl and read at absorbance of 450 nm. The absorbance of each serum dilution was calculated as the absorbance of the antigen lysate–coated wells minus the absorbance of reference lysate-coated wells. A response was defined as positive if the ΔA was greater than the mean ± 2 SD of controls, which are negative for a particular antigen as determined by SEREX.

Analysis of mRNA expression in different tissue and cell lines by real-time reverse transcription-PCR. Total RNA from normal mouse mammary tissue, fresh murine breast tumors, and breast cancer cell lines (MMC and ANV) were isolated using RNA4Aqueous kit. The integrity of RNA was tested using an Agilent BioAnalyzer (Foster City, CA). RNA from normal mouse tissues, including brain, eye, heart, kidney, liver, lung, spleen, testis, thymus, and uterus, was purchased from Clontech (Mountain View, CA). cDNA was generated from 5 μg RNA by SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) with oligo(dT) as primers according to the manufacturer's protocol. Diluted cDNA (5 μL of 1:40) was then used as template for real-time PCR analysis. The primers and probes (FAM-MGB) for Taqman-based gene expression assay were bought from Applied Biosystems (Foster City, CA). Real-time PCR was done in 384-well thin-wall PCR plates using ABI Prism 7900HT under the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and a combined annealing/extension step at 60°C for 1 minute. Data analysis was done using SDS 2.21 (Applied Biosystems). mRNA expression level of tumor antigens was normalized to β-actin using the ΔC_T method. Level of expression = $2^{-\Delta CT}$, where $\Delta C_T = C_{T\ \mathrm{antigen}} - C_{T\ \mathrm{actin}}$. C_T is the cycle threshold at which the fluorescence signal crosses an arbitrary value.

Flow cytometric analysis of tumor-infiltrating immune cells. Immediately after tumor resection, the tumor was minced into small pieces and then immersed in 5 mL digestion mixture (RPMI 1640 with 10% fetal bovine serum, 50 μL of 1% collagenase, and 50 μL of 1% DNase, both are from Life Technologies, Carlsbad, CA). This mixture was incubated on rocker at room temperature for 30 minutes and then filtered through a 70-μm screen. The leukocytes were further enriched by Ficoll. The resulting single-cell suspension was analyzed on a FC500 flow cytometer (Beckman Coulter, Fullerton, CA) after staining with the appropriate antibodies for 30 minutes at 4°C in the dark. All the antibodies (CD3-FITC, CD4-PE, CD8-PerCP, NK1.1-PE, CD19-FITC, and CD11c-PE) were purchased from BD Biosciences (San Diego, CA).

Statistical analysis. χ^2 test was used to compare the frequency of antibody response between tumor group and control group. Student's *t* test was used to compare the amount of infiltrating immune cells in spontaneous versus implanted tumor. SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL) was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

Results

Multiple immunogenic proteins, related to cancer progression, can be identified in tumor-bearing *neu*-tg mice. Immunoscreening of three libraries ($\sim 1 \times 10^6$ phages screened for each library) using sera from mice with spontaneous tumors yielded a total of 62 positive clones. Nucleotide sequence analysis of the cDNA inserts identified 15 different antigens (Table 1). The five mostly frequently identified antigens are the following: cytokeratin 2-8 (Krt2-8; represented by 15 overlapping clones), glutamyl-prolyl-tRNA synthetase (Eprs; 13 clones), complement C3 (C3; 9 clones), galectin 8 (Lgals8; 6 clones), and serine/threonine-rich protein kinase 1 (Srpk1; 5 clones). The antigens are proteins of diverse functions, including structural proteins [Krt2-8, gelsolin (Gsn), and matrin 3 (Matr3)], signal transduction proteins [Rho-associated, coiled coil–containing protein kinase (Rock) 1, Rho/Rac guanine nucleotide exchange factor 2 (Arhgef2), RAB3A-interacting protein (Rab3ip), and schistosoma mansoni adult worm antigen preparation 70 (Swap70)], and transcription factors [leucine zipper transcription factor-like protein 1 (Lztfl1) and Y box-binding protein (Yb1)]. The chrosomal location of the genes did not show any bias toward a specific chromosome (Table 1). In terms of cellular location, only Lgals8 and C3 are extracellular. All the other proteins are intracellular with five nuclear proteins [Yb1, ubiquitin-specific protease 7 (Usp7), Lztfl1, Matr3, and exportin 5 (Xpo5)]. Many of the antigenic proteins play an important role in tumorigenesis. For example, Rab3ip is a proto-oncogene that interacts with the cancer-testis antigen SSX2 (8). Gsn, an actin-severing protein, has tumor suppressor function and has been reported to be down-regulated in human ovarian and breast cancer (9–11). Three of the identified antigens, Rock1, Swap70, and Arhgef2, are involved in the Rho/Rac-Rock signal transduction pathway. Rock1, the effector of RhoA, regulates the organization of the actin cytoskeleton and is responsible for cell motility and cytokinesis (Fig. 1). Arhgef2 is a Rho/Rac guanine nucleotide exchange factor. Swap70 may also function as a guanine nucleotide exchange factor (12). Rho/Rock pathway plays an important role in tumor cell invasion and cancer metastasis (13–17).

SEREX-identified antigens are widely expressed intracellular self-proteins. Real-time reverse transcription-PCR (RT-PCR)

Table 1. Murine TAAs identified by SEREX

Antigen	No. clones	Gene symbol	Chromosomal location	Subcellular location	Function	UniGene no.
Cytokeratin 2-8	15	Krt2-8	15F3	Cytoplasm	Cytoskeleton	Mm.358618
Glutamyl-prolyl-tRNA synthetase	13	Eprs	1H4	Cytoplasm	tRNA aminoacylation for protein translation	Mm.154511
Complement C3	9	C3	17E1	Extracellular	Complement activation	Mm.19131
Galectin 8	6	Lgals8	13A1	Extracellular	Cell adhesion, spreading	Mm.171186
Serine/threonine-rich protein kinase 1	5	Srpk1	17A3	Cytosol/nucleus	Regulate pre-mRNA splicing	Mm.15252
RAB3A-interacting protein	3	Rab3ip	10D2	Cytosol/nucleus	SSX2 interacting protein	Mm.336394
Rho-associated, coiled coil–containing protein kinase 1	2	Rock1	18A2	Cytoplasm	RhoA/Rock signaling pathway, cell differentiation, migration	Mm.6710
Schistosoma mansoni adult worm antigen preparation 70	2	Swap70	7E3	Cytoplasm	Signal transduction, RacGEF	Mm.334144
Rho/Rac guanine nucleotide exchange factor 2	1	Arhgef2	3F1	Cytoplasm	Activation of RhoA/Rock pathway	Mm.239329
Gelsolin	1	Gsn	2B	Cytoplasm	Actin-binding protein, tumor suppressor	Mm.21109
Nuclease-sensitive element binding protein 1 (Y box-binding protein)	1	Nsep1 (Yb1)	4D1	Nucleus	DNA-binding protein	Mm.258204
Ubiquitin-specific protease 7	1	Usp7	16A1	Nucleus	Deubiquitinating enzyme for p53 (39)	Mm.295330
Leucine zipper transcription factor-like protein 1	1	Lztfl1	9F	Nucleus	Transcription factor	Mm.42258
Exportin 5	1	Xpo5	17C	Nucleus	Exports eEF1A via tRNA from nuclei	Mm.275039
Matrin 3	1	Matr3	18C	Nucleus	Nuclear protein	Mm.215034

was used to measure the mRNA expression level of the SEREX-identified antigens in different tissues to examine their expression profile. The expression level was evaluated in a panel of 10 normal tissues, including brain, eye, heart, kidney, liver, lung, spleen, testis, thymus, and uterus. All of the antigens were detected in normal and tumor tissues. Figure 2 shows the expression profile of the five most frequently identified antigens. Krt2-8 is the only antigen that shows relatively high level of expression in testis compared with other normal tissues (Fig. 2*A*). However, it is also expressed in the uterus, kidney, and liver. Srpk1, which was reported previously to have restricted expression in testis (18), was found to express in other tissue as well (Fig. 2*E*). In summary, none of the tumor-associated antigens (TAA) we found showed restricted expression as that of a cancer/testis antigen.

SEREX-identified antigens have dysregulated expression in breast cancer tissue and cell line compared with normal mammary gland. As tumor antigens may be overexpressed in cancer compared with normal tissue, we used real-time RT-PCR to evaluate the expression levels of identified antigens in fresh breast cancers (n = 7), normal mammary tissues (n = 5), and a *neu*-overexpressing cell line, MMC, derived from a spontaneous tumor in the *neu*-tg mouse (5). As shown in Fig. 3, the genes of some antigens are up-regulated in primary tumor and cell line (*Krt2-8* and *Matr3*), whereas other genes (*Gsn, C3*, and *Yb1*) are down-regulated. Similar changes in expression of cytokeratin and Gsn have been reported for human cancer. Cytokeratin is up-regulated in breast cancer (19), especially in *neu*-overexpressing breast cancer (20). Cytokeratin was also overexpressed in hepatocarcinoma, cervical cancer, and renal cancer, similar tissues as we show here in the murine system (21–23). Gsn, as a tumor suppressor, is down-regulated significantly in human breast and ovarian cancers (9, 11, 19, 24).

The majority of identified murine tumor antigens have immunogenic human homologues. To examine if the mouse antigens we identified have immunogenic human homologues, we searched the human cancer immunome database (CIDB; http://www2.licr.org/CancerImmunomeDB/) and PubMed. We found that more than half of the mouse tumor antigens have immunogenic human homologues (Table 2). The relevant cancer types and SEREX clones are listed in Table 2. Two of the proteins involved in the metastatic pathway, Rock1 and Swap70, have been associated with breast and other types of cancer in humans. Furthermore, Rock1 and Srpk1, have been reported to play a role in tumor rejection in multiple myeloma patients (25). Of note, all of the top five most frequently identified antigens have immunogenic human homologues. The seropositivity to these antigens was evaluated in 42 sera from mice bearing spontaneous tumors and 40 sera from control non–tumor-bearing mice using crude lysate ELISA. As shown in the last two columns of Table 2, tumor-bearing mice had significantly higher frequency of antibodies to Krt2-8 and Rock1.

Antibody immunity observed in mice with spontaneous tumors was not observed in mice with implanted tumors. Because implanted tumors are more frequently used as a model system to study tumor immunity, we next questioned if the humoral immunity we observed in mice with spontaneous tumor could also be detected in mice with implanted tumors. Tumor-free *neu*-tg mice received 1×10^6 syngeneic tumor cells (MMC) implanted s.c. All of the *neu*-tg mice developed palpable tumors within 2 weeks after the injection. Serum was collected from *neu*-tg mice before and at 1 month after the tumor implantation to evaluate if new immunity developed associated with tumor growth. In contrast to *neu*-tg mice bearing spontaneous tumors, no mice bearing implanted tumors had evidence of increased antibody

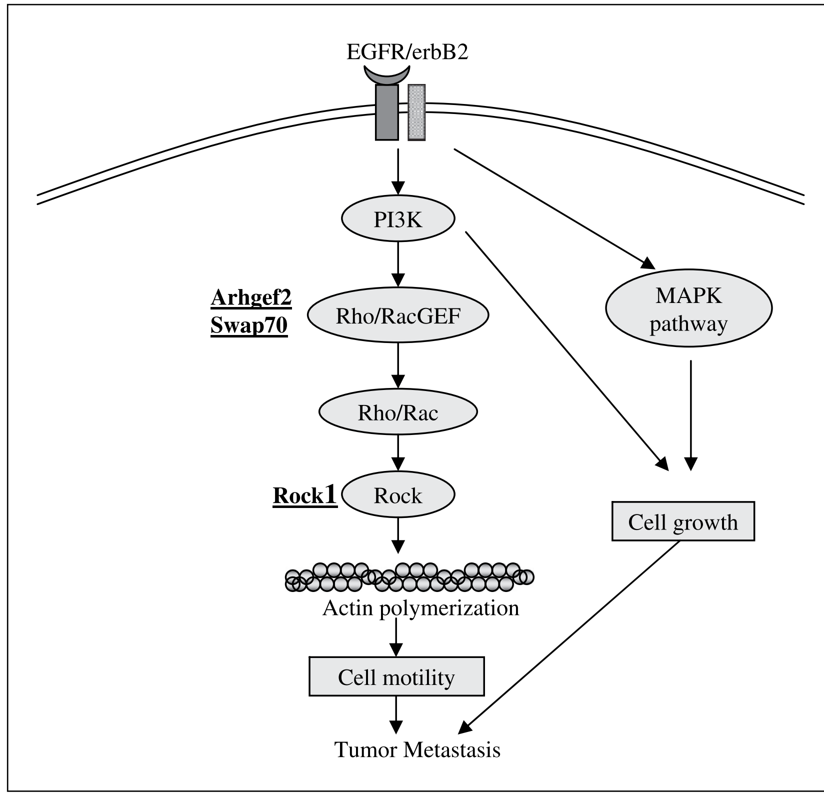

Figure 1. Multiple proteins related to tumor metastasis are found to be immunogenic. Activation of epidermal growth factor receptor (*EGFR*)/erbB2 can lead to actin polymerization and cell motility through the serial activation of Rho/RacGEF, Rho GTPase (Rho/Rac/Cdc42), and Rock. Three proteins involved in this pathway (Arhgef2, Swap70, and Rock1) are found to be immunogenic in *neu*-tg mice. The mitogen-activated protein kinase (*MAPK*) pathway is oversimplified.

response to the most common antigens, cytokeratin, Eprs, Srpk1, C3, Yb1, and Rock1. Figure 4*A* and *B* shows the representative results on cytokeratin, which had increased serum antibodies in mice bearing spontaneous tumor but not in mice with implanted tumor. To further understand the difference between spontaneous and implanted tumors, we compared growth rates. The implanted tumor grew faster than spontaneous tumor (Fig. 4*C*). At 2 weeks after the first appearance of palpable tumors, the average size of a spontaneous tumor was significantly smaller than that of an implanted tumor (274.6 $\pm$ 49.2 versus 1,458.2 $\pm$ 102.6; $P < 0.001$). Furthermore, analysis of immune cell infiltrates in tumor showed that spontaneous tumor had significantly higher numbers of CD4 and CD8 T cells than implanted tumor [6.4 $\pm$ 4.9 versus 2.1 $\pm$ 0.8, CD4 T cells ($P < 0.05$); 4.8 $\pm$ 1.3 versus 1.1 $\pm$ 0.2, CD8 T cells ($P < 0.05$); Fig. 4*D*]. There was no difference for B cells ($CD19^+$), NK cells ($NK1.1^+$), or dendritic cells ($CD11c^+$).

Discussion

We comprehensively identified the immunogenic repertoire of the *neu*-tg mouse. After screening 3 cDNA libraries, we identified 15 tumor antigens with diverse biological functions, including structural proteins, transcription factors, and signal transduction molecules (Table 1). The majority of tumor antigens identified have immunogenic human homologues (Table 2), including Gsn, Rock1, Krt2-8, and Yb1, which have been reported to play important roles in tumorigenesis and metastasis in both human and animal models (9–11, 13–17, 21–25). The dysregulated expression of Krt2-8 and Gsn in breast tumor in *neu*-tg mice also mirrors the expression in cancer patients (9–11, 19, 20). The similarity between mouse and human tumor antigens indicate that *neu*-tg mice may be useful in modeling immunobased therapy for human breast cancer. Moreover, the majority of the tumor antigen repertoire is composed of intracellular proteins. Finally, data presented suggest that the generation of endogenous immunity in mice is associated only with spontaneous tumor development and not the more commonly used therapeutic model system, implanted tumors.

It has long been a matter of debate whether data collected in rodent models would be predictive of human disease response. Indeed, in animal models of tumor immunity, many of the tumor cell lines studied are chemically induced or inherently immunogenic, quite dissimilar to the human condition (26). In particular, the field of tumor immunology has suffered from a lack of success in translating successful murine therapeutic approaches into immunotherapeutics that have an effect in human malignancies. More recently, the ability to generate mice that are transgenic or knocked out for cancer-related genes has resulted in the development of rodent models that closely mimic human cancer from a pathologic standpoint (2, 27–29). Furthermore, a recent review by Roberts et al. (30) showed that transgenic and knockout mouse models could accurately predict some of the toxicities that have been seen in human clinical studies of anticancer agents, such as inhibitors of cyclooxygenase-2, vascular endothelial growth factor, KIT, and ER. *Neu*-tg mice are engineered to express nontransforming rat *neu* on an MMTV promoter and the breast cancers that occur in these mice mimic premenopausal breast cancer in women (2). Gene expression profiling of the mammary tumors in these mice shows that there are numerous similarities to human breast cancer (3). Results presented here show that the majority of tumor antigens identified in *neu*-tg mice have immunogenic human homologues. Thus, the *neu*-tg mouse may serve not only as a potential discovery tool for immunogenic human proteins but also as a model to evaluate the potential therapeutic efficacy of immunologic targeting of those proteins.

In humans, tumor antigens are characterized into different categories, including cancer/testis antigens, overexpressed proteins, differentiation antigens, and mutated proteins to name a few (31). As it is difficult to do comprehensive analyses of the immunologic repertoire in humans, it is unknown which types of proteins are most likely to elicit an immune response in cancer patients. Due to the homogeneity of the *neu*-tg mouse colony, exhaustive immunologic analyses of the antigenic repertoire can be done. Of note, not all mice developed immunity to their tumors, and not all animals developed immunity to the same antigens. Furthermore, many mice developed immunity to multiple antigens so the generation of immunity was heterogeneic despite the genetic similarity of the breast cancers arising in these animals. With one exception of finding a possible cancer/testis antigen, our studies show that the most common similar characteristic of all identified antigens was an intracellular location. Intracellular self-proteins are not normally recognized by the immune system due to immunologic ignorance. In disease conditions, such as cancer or other trauma, the proteins are made visible to the immune system due to tissue destruction. In addition, results presented here suggest that activation of metastatic pathways may make a protein immunogenic. The fact that 3 of the 15 identified antigens fall in the Rho/Rock signal transduction pathway support this hypothesis. Our results support the hypothesis that the immunogenicity of a

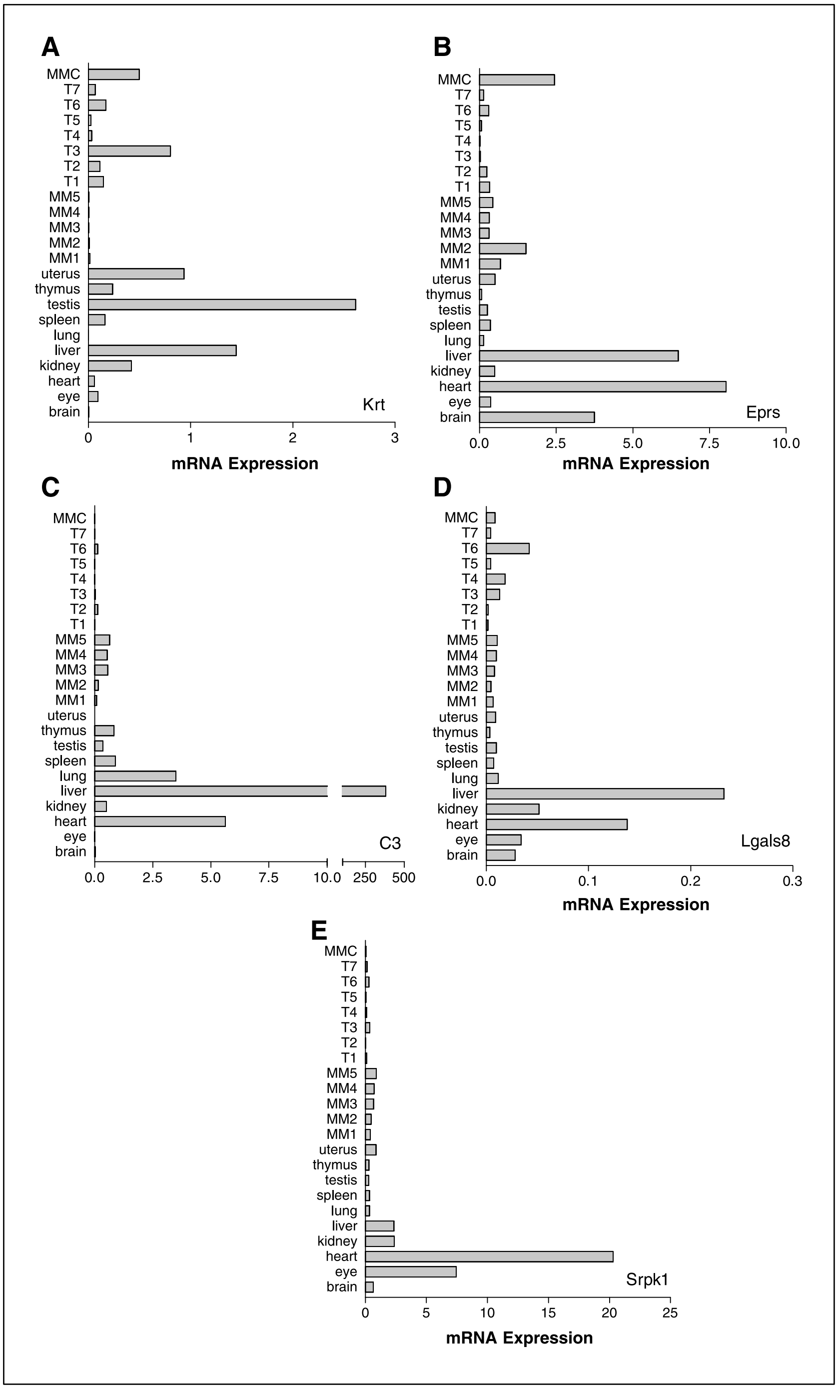

Figure 2. SEREX-identified antigens are widely expressed intracellular self-proteins. Real-time RT-PCR analyses measuring the mRNA expression levels of the top five most frequently identified TAAs. *A,* Krt2-8; *B,* Eprs; *C,* C3; *D,* Lgals8; *E,* Srpk1. *MM1* to *MM5,* five mouse mammary specimen; *T1* to *T7,* seven mouse tumor specimen from the *neu*-tg mice. The mRNA expression level of each TAA is normalized to β-actin.

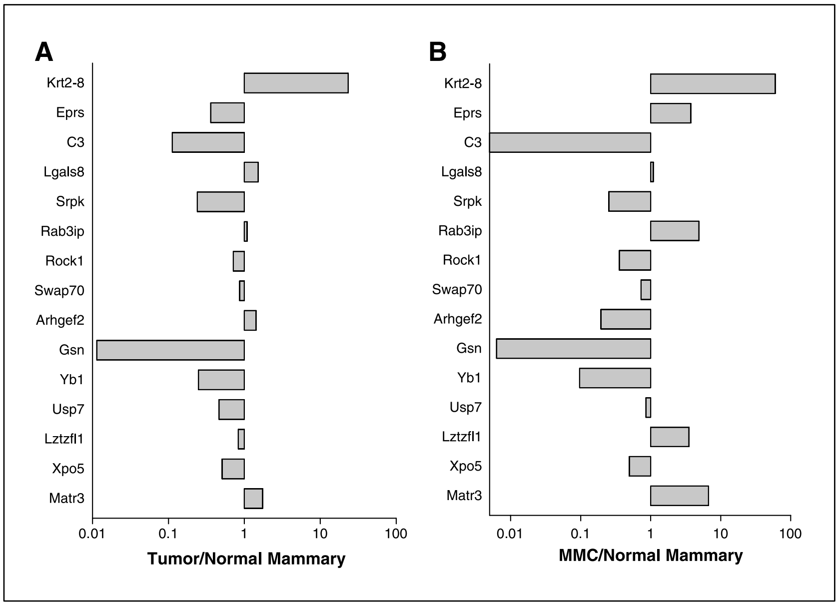

Figure 3. SEREX-identified TAAs may be up-regulated or down-regulated in breast tumor and cell lines. The mRNA expression level of each TAA is normalized to β-actin. Ratio of relative expression level of tumor to normal (*A*) or cell lines to normal mammary (*B*). Ratio of the average expression level in 7 tumors and 5 mammary tissues (*A*).

given molecule depends on the context, in which it is presented than on its more or less restricted expression in certain tissues (32).

SEREX can identify not only new therapeutic targets but also new diagnostic markers (33). In the present study, we examined the presence of antibodies to SEREX-identified antigens in 42 sera from tumor-bearing animal and 40 controls. The results showed that tumor-bearing mice had significantly higher frequency of antibody responses to Krt2-8 and Rock1. Krt2-8 is a component of the intracellular cytoskeleton in cells of the single-layered sheet tissues. It was overexpressed in both tumor and a *neu*-positive cell line from *neu*-tg mice (Fig. 3). It is also reported to be overexpressed in cancer patients (19–23). Rock1 is the downstream effector of Rho and regulates actin polymerization and focal adhesion (Fig. 1). The Rho/Rock pathway plays an essential role in tumor metastasis (13–17). Whether cytokeratin or Rock1 could be potential new diagnostic markers needs more investigation. To examine if any of the antigens may be used as potential therapeutic targets, we evaluated the antibody response after successful immunomodulation, which may result in the development of new tumor-specific antibody immune responses that correlate with tumor regressions. We treated *neu*-tg mice with a reagent to deplete T regulatory cells, an anti-CD25 immunotoxin, and the spontaneous tumors

Table 2. Murine TAAs that have immunogenic human homologues

Mouse TAA	Human homologue	Cancer types	References	SEREX clone in CIDB*	Murine serum reactivity	
					Tumor	Control
Krt2-8	KRT2-8	Ovarian, colon, renal	CIDB (40)	MO-OVA-122	15/42	5/40 †
Eprs	EPRS	Breast, colon, gastric	(41)	N/A	7/42	2/40
C3	C3	Head, neck	CIDB	AU-HN-4	14/42	6/40
Lgals8	LGALS8	Prostate	(42)	N/A	14/42	13/40
Srpk1	SRPK1	Breast, leukemia	CIDB (25, 43)	NGO-BR-51	7/42	3/40
Rock1	ROCK1	Breast, renal, fibrosarcoma, myeloma	CIDB (25)	NY-REN-35 NGO-BR-4 NW-TK156 NY-SAR-66	6/42	0/40 †
Swap70	SWAP70	Breast, melanoma, renal, lymphoma	CIDB	HOM-HD2-117 HOM-Mel2-2.7 MO-BC-1046 MO-REN-15 NGO-BR-44 NY-REN-5	6/42	5/40
Nsep1 (Yb1)	NSEP1 (YB1)	Breast, ovarian	CIDB (44)	HOM-Ts-PMR2-12 HOM-TSOv2-35	4/42	0/40

*Human CIDB (http://www2.licr.org/CancerImmunomeDB/).
†$P < 0.05$, χ^2 test.

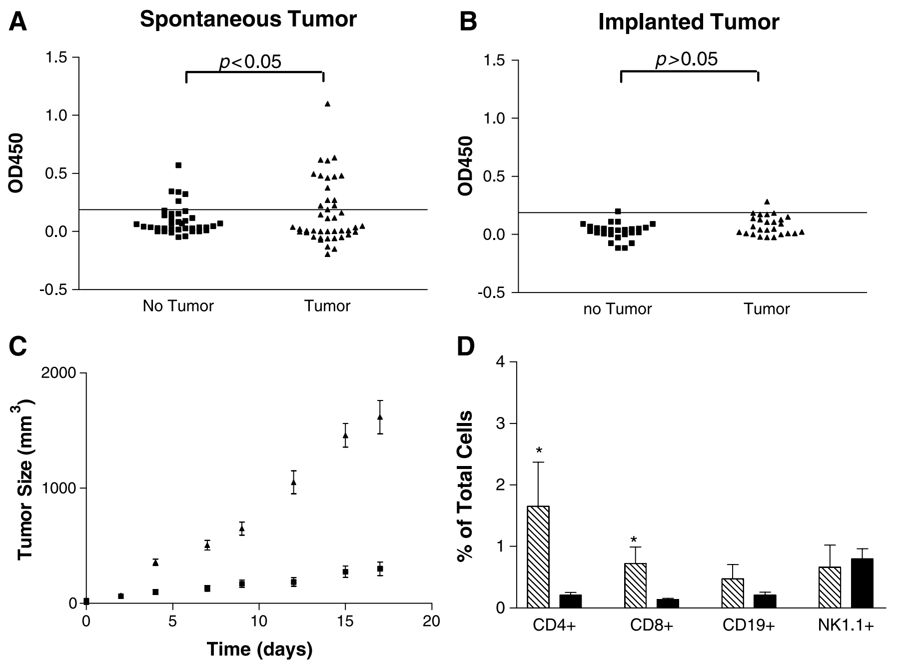

Figure 4. Antibody immunity observed in mice with spontaneous tumor was not observed in mice with implanted tumor. *A,* representative ELISA result showing the antibody response to Krt2-8 in sera from mice with spontaneous tumor (▲; $n = 42$) and control mice (■; $n = 40$). ΔA for each individual sample. *Solid line,* mean ± 2 SDs of 10 serum samples that were tested to be negative for the Krt2-8 antibody by SEREX. *B,* antibody response to Krt2-8 in sera collected from *neu*-tg mice before and after tumor implantation ($n = 26$). *C,* growth curve of spontaneous (■) and implanted (▲) tumors. *Points,* mean of 10 mice; *bars,* SE. *X axis,* days after the first appearance of palpable tumors. *D,* percentage of tumor-infiltrating immune cells as determined by flow cytometric analyses. *Slashed columns,* cells in spontaneous tumors; *solid columns,* cells in implanted tumors. *, ($P < 0.05$), a significant difference between the spontaneous and implanted tumors.

regressed. Both anti-*neu* antibody and T-cell immunity was augmented after the treatment. We did ELISA using pretumor and post-tumor regression sera and found that antibody immunity to one of the SEREX-identified antigens, Srpk1, increased post-tumor regression (34). It is interesting that patients with relapsed myeloma developed new antibody immunity to Srpk1 after complete tumor regression was induced with donor lymphocytes infusions (25).

Tumor implants have long been used to evaluate immunobased strategies in rodent models. Our studies show that there are significant differences in the immune responses that occur endogenously in animals that spontaneously developed tumor versus received s.c. tumor implants. A humoral immune response was only increased in transgenic mice that developed spontaneous tumors. Moreover, the magnitude and phenotype of infiltrating cells were significantly different between spontaneous and implanted tumors. Several factors could contribute to this finding. First, the growth rate of implanted tumors is accelerated, which may impede the development of immunity. Second, the tumor implants are in a foreign anatomic site, which may affect the ability of initiating an immune response. Furthermore, implanted tumors generally do not have associated stromal elements, which are critical in the generation of immunity (35, 36). The cell line (MMC) used for the implanted tumor was derived from a spontaneous tumor in *neu*-tg mice. Previous work in our laboratory has shown that antigen loss variant may develop from this cell line when implanted in mice (37, 38). Therefore, we examined the rat *neu* expression in the dissected implanted tumor. The expression of rat *neu* remained high in those tumors (data not shown), indicating the absence of antibody response was not due to antigen loss. Data presented here would support the use of animal models, which develop spontaneous tumors as an experimental system that more closely resembles endogenous immunity occurring in human tumors (26).

High-throughput methods, such as SEREX and other proteomic techniques, have resulted in the identification of a multitude of immunogenic targets. The critical block in exploiting these targets for therapeutic use to affect human disease is determining which proteins have clinical importance (i.e., tumor rejection antigens). Data presented here suggest that, at least for ER-negative breast cancer, the *neu*-tg mouse may be an ideal model for prioritization of therapeutic immunologic targets due to the high degree of homology with the immunologic signature of human cancers.

Acknowledgments

Received 3/23/2006; revised 7/25/2006; accepted 7/31/2006.

Grant support: NIH grants R01 CA101190 and K24 CA85218 (M.L. Disis).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Brad Stone for constructive discussions.

References

1. Jager D, Taverna C, Zippelius A, Knuth A. Identification of tumor antigens as potential target antigens for immunotherapy by serological expression cloning. Cancer Immunol Immunother 2004;53:144–7.
2. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992;89:10578–82.
3. Andrechek ER, Laing MA, Girgis-Gabardo AA, Siegel PM, Cardiff RD, Muller WJ. Gene expression profiling of *neu*-induced mammary tumors from transgenic mice reveals genetic and morphological similarities to ErbB2-expressing human breast cancers. Cancer Res 2003;63:4920–6.
4. Bershtein LM, Alimova IN, Tsyrlina EV, Anisimov VN. Mammary tumors in HER-2/*neu* mice are characterized by low content of estrogen receptors-α and absence of progesterone receptors. Bull Exp Biol Med 2003;135:580–1.

5. Manjili MH, Arnouk H, Knutson KL, et al. Emergence of immune escape variant of mammary tumors that has distinct proteomic profile and a reduced ability to induce "danger signals." Breast Cancer Res Treat 2005;96:233–41.
6. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A 1995;92: 11810–3.
7. Tureci O, Luxemburger U, Heinen H, et al. CrELISA: a fast and robust enzyme-linked immunosorbent assay bypassing the need for purification of recombinant protein. J Immunol Methods 2004;289:191–9.
8. de Bruijn DR, dos Santos NR, Kater-Baats E, et al. The cancer-related protein SSX2 interacts with the human homologue of a Ras-like GTPase interactor, RAB3IP, and a novel nuclear protein, SSX2IP. Genes Chromosomes Cancer 2002;34:285–98.
9. Asch HL, Head K, Dong Y, et al. Widespread loss of gelsolin in breast cancers of humans, mice, and rats. Cancer Res 1996;56:4841–5.
10. Sagawa N, Fujita H, Banno Y, Nozawa Y, Katoh H, Kuzumaki N. Gelsolin suppresses tumorigenicity through inhibiting PKC activation in a human lung cancer cell line, PC10. Br J Cancer 2003;88:606–12.
11. Noske A, Denkert C, Schober H, et al. Loss of gelsolin expression in human ovarian carcinomas. Eur J Cancer 2005;41:461–9.
12. Shinohara M, Terada Y, Iwamatsu A, et al. SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. Nature 2002; 416:759–63.
13. Croft DR, Sahai E, Mavria G, et al. Conditional ROCK activation *in vivo* induces tumor cell dissemination and angiogenesis. Cancer Res 2004;64:8994–9001.
14. Kamai T, Tsujii T, Arai K, et al. Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 2003;9:2632–41.
15. Kamai T, Yamanishi T, Shirataki H, et al. Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. Clin Cancer Res 2004;10:4799–805.
16. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 1999;5:221–5.
17. Wang DS, Dou KF, Li KZ, Song ZS. Enhancement of migration and invasion of hepatoma cells via a Rho GTPase signaling pathway. World J Gastroenterol 2004; 10:299–302.
18. Papoutsopoulou S, Nikolakaki E, Chalepakis G, Kruft V, Chevaillier P, Giannakouros T. SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1. Nucleic Acids Res 1999;27:2972–80.
19. Somiari RI, Sullivan A, Russell S, et al. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. Proteomics 2003;3: 1863–73.
20. Zhang DH, Tai LK, Wong LL, Sethi SK, Koay ES. Proteomics of breast cancer: enhanced expression of cytokeratin 19 in human epidermal growth factor receptor type 2 positive breast tumors. Proteomics 2005;5:1797–805.
21. Srisomsap C, Sawangareetrakul P, Subhasitanont P, et al. Proteomic analysis of cholangiocarcinoma cell line. Proteomics 2004;4:1135–44.
22. Carrilho C, Alberto M, Buane L, David L. Keratins 8, 10, 13, and 17 are useful markers in the diagnosis of human cervix carcinomas. Hum Pathol 2004;35:546–51.
23. Kellner R, Lichtenfels R, Atkins D, et al. Targeting of tumor associated antigens in renal cell carcinoma using proteome-based analysis and their clinical significance. Proteomics 2002;2:1743–51.
24. Winston JS, Asch HL, Zhang PJ, Edge SB, Hyland A, Asch BB. Downregulation of gelsolin correlates with the progression to breast carcinoma. Breast Cancer Res Treat 2001;65:11–21.
25. Bellucci R, Wu CJ, Chiaretti S, et al. Complete response to donor lymphocyte infusion in multiple myeloma is associated with antibody responses to highly expressed antigens. Blood 2004;103:656–63.
26. Ostrand-Rosenberg S. Animal models of tumor immunity, immunotherapy, and cancer vaccines. Curr Opin Immunol 2004;16:143–50.
27. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 1995;92:3439–43.
28. Clarke P, Mann J, Simpson JF, Rickard-Dickson K, Primus FJ. Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy. Cancer Res 1998;58:1469–77.
29. Wang S, Gao J, Lei Q, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell 2003;4:209–21.
30. Roberts RB, Arteaga CL, Threadgill DW. Modeling the cancer patient with genetically engineered mice: prediction of toxicity from molecule-targeted therapies. Cancer Cell 2004;5:115–20.
31. Mocellin S, Mandruzzato S, Bronte V, Lise M, Nitti D. Part I: vaccines for solid tumours. Lancet Oncol 2004;5: 681–9.
32. Sahin U, Tureci O, Pfreundschuh M. Serological identification of human tumor antigens. Curr Opin Immunol 1997;9:709–16.
33. Zhong L, Peng X, Hidalgo GE, Doherty DE, Stromberg AJ, Hirschowitz EA. Antibodies to HSP70 and HSP90 in serum in non-small cell lung cancer patients. Cancer Detect Prev 2003;27:285–90.
34. Knutson KL, Dang Y, Lu H, et al. IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in *neu*-transgenic mice. J Immunol 2006; 177:84–91.
35. Spiotto MT, Yu P, Rowley DA, et al. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. Immunity 2002;17:737–47.
36. Blankenstein T. The role of tumor stroma in the interaction between tumor and immune system. Curr Opin Immunol 2005;17:180–6.
37. Knutson KL, Lu H, Stone B, et al. Immunoediting of cancers may lead to epithelial to mesenchymal transition. J Immunol 2006;177:1526–33.
38. Knutson KL, Almand B, Dang Y, Disis ML. *Neu* antigen-negative variants can be generated after *neu*-specific antibody therapy in *neu* transgenic mice. Cancer Res 2004;64:1146–51.
39. Lim SK, Shin JM, Kim YS, Baek KH. Identification and characterization of murine mHAUSP encoding a deubiquitinating enzyme that regulates the status of p53 ubiquitination. Int J Oncol 2004;24:357–64.
40. Ditzel HJ, Strik MC, Larsen MK, et al. Cancer-associated cleavage of cytokeratin 8/18 heterotypic complexes exposes a neoepitope in human adenocarcinomas. J Biol Chem 2002;277:21712–22.
41. Line A, Slucka Z, Stengrevics A, Silina K, Li G, Rees RC. Characterisation of tumour-associated antigens in colon cancer. Cancer Immunol Immunother 2002;51: 574–82.
42. Su ZZ, Lin J, Shen R, Fisher PE, Goldstein NI, Fisher PB. Surface-epitope masking and expression cloning identifies the human prostate carcinoma tumor antigen gene PCTA-1 a member of the galectin gene family. Proc Natl Acad Sci U S A 1996;93:7252–7.
43. Hishizawa M, Imada K, Sakai T, Ueda M, Hori T, Uchiyama T. Serological identification of adult T-cell leukaemia-associated antigens. Br J Haematol 2005;130: 382–90.
44. Stone B, Schummer M, Paley PJ, et al. Serologic analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q. Int J Cancer 2003;104:73–84.

The Identification of Tumor Rejection Antigens in Murine Models That are Associated with Human Homologues

Hailing Lu, Ekram Gad, Amy Chang, Kristin Seymour, and Mary L. Disis

Tumor Vaccine Group, University of Washington, Seattle, WA 98195

There are many discovery tools that are useful for the identification of human tumor antigens. Although hundreds of human tumor antigens have been identified, it is unknown which of those proteins is a true “tumor rejection” antigen. We questioned whether murine models could be used to predict human tumor rejection antigens. Neu-tg FVB/N mice which express rat neu on an MMTV promoter develop HER2+ER− breast cancer that mimics premenopausal human breast cancer. Using sera from mice bearing spontaneous tumors, we identified 16 mouse tumor antigens through SEREX (serological analysis of recombinant cDNA expression libraries). Half of the mouse TAA (tumor associated antigens) have immunogenic human homologues, which suggest that the neu-tg mice could be used to predict human TAAs. To further identify antigens that are associated with tumor rejection, we established a tumor rejection model by implanting *in vitro* cultured HER2+ mouse mammary carcinoma (MMC) cells derived from a spontaneous tumor into parental FVB/N mice. While $2x10^6$ MMC implantation resulted in 100% tumor development in neu-tg mice within 2 weeks, the parental mice were able to completely reject the tumor implant. Anti-neu antibody was detected in the serum from parental mice after they rejected neu+ tumor cells using flow cytometry. Adoptive transfer of splenocytes from the tumor rejection mice to neu-tg mice resulted in inhibited tumor growth in the recipient mice. We hypothesized that the tumor rejection effect observed in parental mice was not only due to immune response against neu which is a foreign protein, but also against other tumor antigens which could be detected by SEREX. Blood was taken from the parental mice before and after tumor rejection. Subtractive SEREX was used to search for post-rejection specific antibodies. The first antigen we identified is alpha catenin (Catnna1), which is a cadherin binding protein that is involved in cell adhesion and tumor metastasis. After demonstrating novel polyclonal immune responses could be generated with tumor regression in parental mice, we evaluated if polyclonal immunity was also generated in neu-tg mice after immune based therapies which resulted in tumor regression. Sera samples from transgenic mice before and after immunomodulation were collected to determine the presence of new immunity associated with tumor regression. Preliminary analysis showed that new immunity to a mouse tumor antigen serine/arginine-rich protein specific kinase 1 (Srpk1) developed after therapeutically effective immumodulation. The “immunologic signature” of tumor rejection antigens in neu transgenic mice will be presented.

Identification of an immunological signature of tumor rejection in the neu-transgenic mouse

Hailing Lu, Amy Chang, Ekram Gad, Emily Larson, and Mary L. Disis

Tumor Vaccine Group, University of Washington, Seattle, WA 98195

With a multitude of human tumor antigens identified to date, the challenge lies in identifying those antigens that, when immunologically targeted, would result in tumor rejection. Previous work from our group has shown that neu-transgenic (neu-tg) mice bearing spontaneous tumors have endogenous immune responses against their tumor. Furthermore, the tumor antigen repertoire identified in neu-tg mice may predict human tumor antigens. In the current study, two tumor rejection models in neu-tg mice were established; (1) parental mice rejecting tumor cell implants, and, (2) tumor regression in neu-tg mice after immunomodulation. Serum samples were collected from mice before and after tumor rejection. Subtractive serological screening of cDNA expression library (SEREX) was used to profile the change in serum antibodies after tumor rejection. After screening 2 cDNA expression libraries established using RNA from spontaneous tumor and a syngeneic tumor cell line, we identified 10 antigens whose antibody responses were preferentially observed in post-rejection sera, including centrosomal protein 290 (Cep290), catenin alpha 1 (Ctnna1), FXYD domain containing ion transport regulator 3 (Fxyd3), GPI-anchored membrane protein 1 (GPIap1), heat shock protein 40 (Hsp40), heterogeneous nuclear ribonuclear protein L-like (Hnrpll), mouse mammary tumor virus 1 (Mtv1), talin 1 (Tln1), tumor necrosis factor alpha inducible protein 3 (Tnfaip3), and transmembrane protein 57 (Tmem57). Four of these antigens have immunogenic human homologues. This antigenic repertoire is distinct from the repertoire we previously identified in tumor-bearing mice. Preliminary data has shown that vaccination targeting Mtv1, a tumor rejection antigen, has a tumor protective effect. Thus, tumor rejection antigens can be identified in neu-tg mice and may serve as a model for identifying human tumor antigens which should be evaluated for immunologic targeting.

Vaccination targeting antigens identified in tumor rejection mice but not antigens identified in tumor bearing mice has tumor protective effect

Hailing Lu, Amy Chang, Ekram Gad, Emily Larson, Eddy Park, Steven La, and Mary L. Disis

Tumor Vaccine Group, University of Washington, Seattle, WA 98195

With a multitude of human tumor antigens identified to date, the challenge lies in identifying those antigens that, when immunologically targeted, would result in tumor rejection. Previously we have shown that neu-transgenic (neu-tg) mice bearing spontaneous tumors have endogenous immune responses against their tumor. A panel of 15 tumor antigens was identified by serological screening of cDNA expression library (SEREX) using sera from tumor bearing mice. Approximately 70% of the tumor antigens identified in neu-tg mice have immunogenic human homologues. We examined the potential therapeutic value of this panel of antigens by vaccinating the mice using plasmids encoding the individual antigens. Unfortunately, none of the tested antigens showed tumor protective effect. We hypothesized that tumor rejection mice may be more suitable than tumor bearing mice in identifying antigens that are associated with tumor rejection. To test this hypothesis, we established two mouse tumor rejection models; (1) parental mice rejecting tumor cell implants; and, (2) tumor regression in neu-tg mice after immunomodulation. Serum samples were collected from mice before and after tumor rejection. Subtractive serological screening of cDNA expression library (SEREX) was used to profile the change in serum antibodies after tumor rejection. After screening 2 cDNA expression libraries, we identified 10 antigens whose antibody responses were preferentially observed in post-rejection sera, including centrosomal protein 290 (Cep290), catenin alpha 1 (Ctnna1), FXYD domain containing ion transport regulator 3 (Fxyd3), GPI-anchored membrane protein 1 (GPIap1), heat shock protein 40 (Hsp40), heterogeneous nuclear ribonuclear protein L-like (Hnrpll), mouse mammary tumor virus 1 (Mtv1), talin 1 (Tln1), tumor necrosis factor alpha inducible protein 3 (Tnfaip3), and transmembrane protein 57 (Tmem57). Four of these antigens have immunogenic human homologues. This antigenic repertoire is distinct from the repertoire we previously identified in tumor bearing mice. Preliminary data has shown that vaccination targeting Mtv1 and Fxyd3 has tumor protective effect. Thus, Vaccination targeting antigens identified in tumor rejection mice but not antigens identified in tumor bearing mice has tumor protective effect.

Preventing the development of breast cancer by immunizing with multi-antigen vaccines targeting proteins associated with oncogenesis. M.L. Disis, E. Gad, D. Cecil, K. Park, V. Lai, R. Lubet*, and H. Lu. Tumor Vaccine Group, University of Washington, Seattle, WA and *Cancer Chemoprevention Branch, National Cancer Institute, Bethesda, MD.

Vaccines have been extremely successful in the prevention of infectious diseases. Cancer vaccines have long been studied for the treatment of cancer, but have not yet been exploited as chemoprevention agents with the exception of virally mediated malignancies. Human breast cancer is immunogenic and a variety of proteins involved in the malignant transformation or maintenance of the malignant phenotype have been identified as tumor antigens. Transgenic animal models are uniquely suited for evaluating the potential efficacy of preventative cancer vaccines. TgMMTV-neu mice, for example, develop neu-mediated breast cancer at about 100 days of life. Tumors are histologically similar to human breast cancer and have a molecular profile consistent with the luminal subtype. We have developed two multi-antigen vaccines targeting immunogenic proteins associated with oncogenesis and evaluated whether we could prevent the development of breast cancer in TgMMTV-neu mice. Each vaccine was composed of 3 different antigens. Class II epitopes derived from antigenic proteins involved in growth factor pathways comprised one vaccine and the other vaccine was plasmid based and encoded intracellular immunogenic proteins involved in cell proliferation. The ability of active immunization to prevent breast cancer was evaluated in both older (18 week) and younger (5 week) mice. Vaccines were administered intradermally every two weeks for 3 immunizations and then monthly until the development of disease or 1 year had passed. Both vaccines prevented breast cancer development in older animals. 40% of peptide immunized older mice were cancer free at 1 year compared to 5% of controls ($p=0.02$). The DNA vaccine appeared to be more effective in older animals with 60% of vaccinated mice tumor free as compared to 20% of controls ($p=0.01$). Protection from the development of cancer was mediated by T cells as evidenced by adoptive transfer of $CD3^+$ cells purified from vaccinated and protected mice, sham immunized mice, and naïve mice. T cells from protected mice resulted in significant inhibition of tumor growth after a syngeneic tumor challenge ($p=0.01$ protected vs. sham vaccine). The cancer protective effect of immunization was more pronounced when vaccines were administered to the younger mice. The Class II epitope vaccine prevented cancer development in 65% of mice as compared to 5% of controls. The plasmid based vaccine protected 70% of mice from breast cancer. Active immunization was associated with a survival benefit in all groups. These data suggest breast cancer vaccines targeting biologically relevant proteins may be an effective approach to the prevention of breast cancer.